Characterisation of biofilm associated with bacterial vaginosis

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Karakterisering van biofilm geassocieerd met bacteriële vaginose

Voor mama

Colophon

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Characterisation of biofilm associated with bacterial vaginosis

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"If you don't like bacteria, you're on the wrong planet."

– Stewart Brand

Dankwoord

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Abbreviations

AEEA	8-amino-3,6-dioxaoctanoicacid
ARDRA	amplified ribosomal DNA restriction analysis
BAP	biofilm-associated protein
BV	bacterial vaginosis
BVAB	BV-associated bacterium
CDC	Centers for Disease Control and Prevention
cfu	colony forming units
CLASI	combinatorial labelling and spectral imaging
CVF	cervicovaginal fluid
CVR	contraceptive vaginal ring
DAPI	6-diamidine-2-phenylindole dihydrochloride
DGGE	denaturing gradient gel electrophoresis
eDNA	extracellular DNA
EPS	extracellular polymeric substances
FISH	fluorescence in situ hybridisation
geq	genome equivalents
HPLC	high performance liquid chromatography

hsp70	heat shock protein 70
HSV-2	herpes simplex virus type 2
IgA	immunoglobuline A
IVR	intravaginal ring
LEA	lauramide arginine ethyl ester
NGS	next-generation sequencing
NO	nitric oxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PID	pelvic inflammatory disease
PNA	peptide nucleic acid
qPCR	quantitative real-time PCR
RAPD	random amplified polymorphic DNA
ROS	reactive oxygen species
STI	sexually transmitted infections
UTI	urinary tract infection

CHAPTER 1

General introduction

The lower reproductive tract is a highly versatile part of the female reproductive system, populated by a range of bacterial species that can have a profound effect on the health of women and their newborns as opposed to a mere passageway for menstrual fluid, sperm and neonates.

We have knowledge of these bacterial species, at least of the most important ones — or should we say "the most abundant ones"? Or even "the ones that are the easiest to cultivate"? A substantial percentage of microorganisms found in the human body are not cultivable using standard techniques [1], so there is a very good chance we are missing out on more than a few of the vaginal bacteria. To deal with this limitation, an increasing range of culture-independent methods is being developed and deployed, revealing more and more detail of the vaginal microbiome. In spite of that, more research is needed to fully understand the ins and outs of the vaginal environment.

When I first started studying the vaginal microbiome, while coordinating the "Microbicides Biomarkers" study in Africa [2], I became intrigued by the high prevalence of bacterial vaginosis (BV) in this representative cohort of African women. Moreover, this problem was not unique for the women residing in sub-Saharan Africa: we also found a BV prevalence of 30% in women visiting a local sexually transmitted infections (STI) clinic and HIV testing and counselling centre in Antwerp, Belgium [3]. Additionally, a small number of young Belgian adolescent girls, another group whose vaginal microbiome we studied in the previous years, were also diagnosed with BV [4]. It was fascinating that BV was common in both high-risk women and young adolescent girls.

BV is a major cause of preterm birth and increases the possibility of getting infected with STIs [5]. This is an immense problem, especially in the group of women at increased risk for STIs, not coincidentally the group of women in whom BV is most prevalent [6].

Effective treatment and prevention for BV are still beyond our reach, unfortunately. Currently available antibiotics can relieve the symptoms temporarily, but after a while the bacteria revive and recolonise the vagina, causing recurrent symptoms. This recurrence of symptoms is typical of chronic infections and it has been hypothesised that the development of a biofilm is at the root of this process. The biofilm creates a safe harbour for non-commensal bacteria [7]. The bacteria in this biofilm are sticking together in a self-produced matrix and are less sensitive to the effects of antibiotic therapy and the host immune system [8].

Very limited research has been performed on this BV-associated biofilm. The research group of Swidsinki [9] was one of the first in the world, and certainly the first group in Europe, to study the vaginal biofilm and bring new techniques such as fluorescence in situ hybridisation (FISH) into the field of BV research. I considered myself very lucky to be able to visit this group, to get trained in FISH, and to be able to discuss this intriguing concept with dr. Swidsinski in person. From there on, I tried to ameliorate the FISH technique, studying not only *Gardnerella vaginalis*, but also its apparent partner in crime: *Atopobium vaginae*. I employed a more stable type of probes and fluorophores, providing me with clear images of the biofilm attaching to the vaginal epithelium. We used this technique and other molecular methods at the Institute of Tropical Medicine (ITM) to try and understand why *G. vaginalis* can be detected in the vaginal environment of women with BV, as well as in women without any signs or symptoms of a vaginal imbalance.

I applied the developed techniques to samples of the Ring Plus study. This study, coordinated by the ITM, involved a group of Rwandan women with a high BV prevalence who were introduced to the concept of vaginal rings and were enrolled to use contraceptive vaginal rings for three months. We considered it would be important, next to knowing how these women perceived the vaginal rings, to study the effect of these rings on the vaginal microbiome and vice versa. Once they hit the market, vaginal rings should be safe in these highly vulnerable populations, considering future possible applications of the rings for prevention of HIV and treatment of STIs.

I was able to study the BV-associated biofilm and its effect on contraceptive vaginal rings in the STI/HIV Reference Laboratory at the ITM, in collaboration with the Laboratory Bacteriology Research at Ghent University under the guidance of my promotors, all three of them specialists in their own discipline, working together for a common cause. I am truly proud to be able to present the result of all of this hard work.

CHAPTER 2

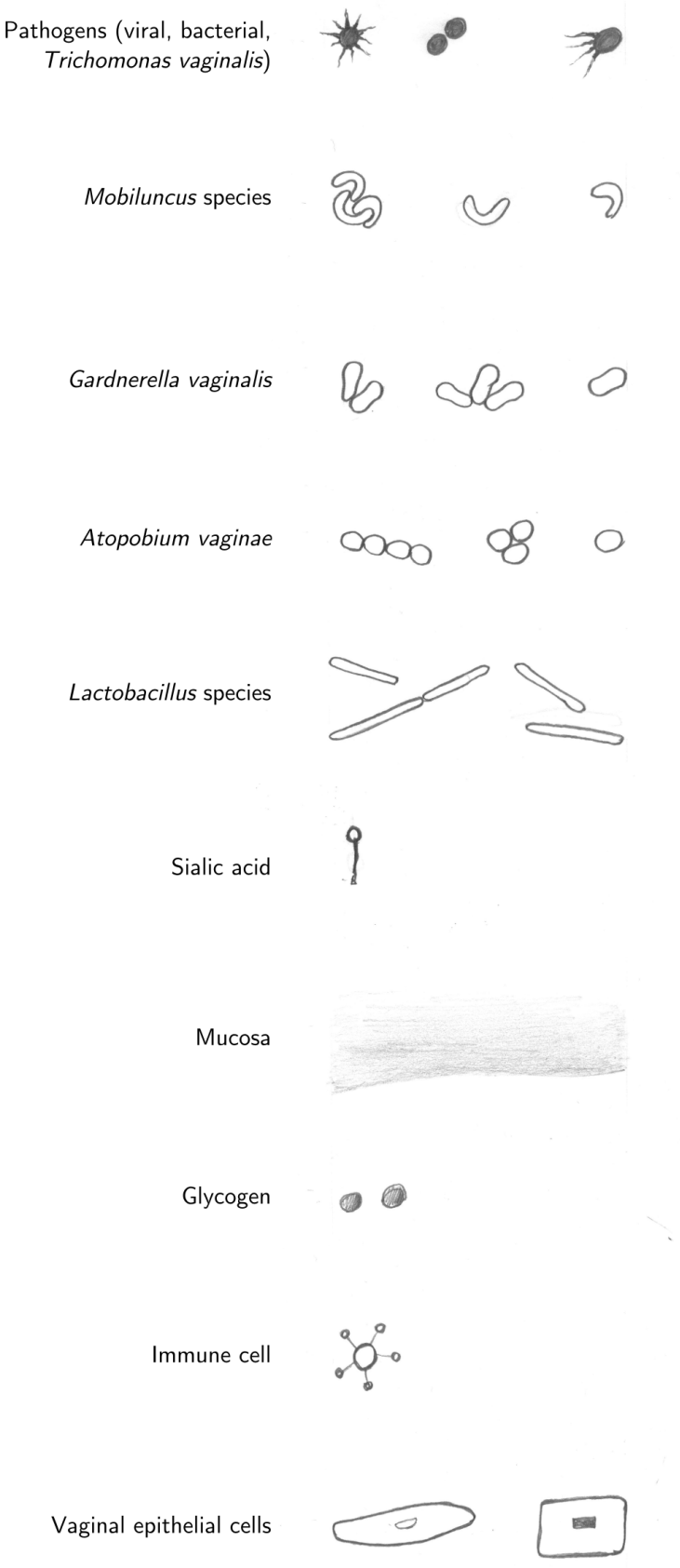
State of the art

The state of the vaginal environment affects the likelihood of conception, the probability of a successful pregnancy and the risk of acquiring sexually transmitted infections (STIs). The healthy vaginal epithelium serves as a highly protective barrier against STIs in sexually active women. The vagina consists of a stratified squamous epithelium of about 28 layers overlying a loose connective tissue stroma [10]. Apical epithelial cells are covered by a glycocalyx layer that hydrates the luminal surface and may act to prevent microbial attachment [11]. Also, the stratum corneum on the luminal surface consists of several layers of dead cells that, besides being uninfectable, are shed continuously (each four hours one cell layer is lost [10, 12]), thereby reducing the ability of pathogens to migrate deeper into the epithelium. However, disruptions in this protective layer could facilitate the invasion of pathogens [13].

2.1 The healthy vaginal ecosystem

Before the establishment of molecular techniques, the definition of a healthy vaginal environment was based on the absence of vaginal complaints, mainly by having a low vaginal pH and not having vaginal discharge or other symptoms of inflammation. However, since the introduction of molecular techniques, it became apparent that there is no such thing as a single "healthy" vaginal microbiome [14]. It has been shown that lactobacilli are predominantly present in the vaginal microbiomes of healthy women of reproductive age [3, 15–18]. However, a considerable percentage of women with an apparent healthy

Figure 2.1: Legend of elements used in the following figures



vaginal microbiome (i.e. without symptoms of a vaginal disturbance) also have non-*Lactobacillus*-species in their microbiome, e.g. *Gardnerella vaginalis*, *Atopobium vaginae*, *Prevotella* spp., *Streptococcus* spp., *Staphylococcus* spp. and *Escherichia coli* [19].

2.1.1 Composition of the cervicovaginal fluid

The vaginal epithelium is kept moist by a cervicovaginal fluid (CVF) that is a mixture of plasma transudate percolating through the vaginal wall and endocervical mucus produced by goblet cells [20]. In normal circumstances, outside of coitus, the CVF also contains mucous secretions from Bartholin's and Skene's glands, exfoliated epithelial cells, residual urine, and fluids from the upper reproductive tract such as endometrial and tubal fluids. The exact composition of the CVF is variable and depends on the levels of the hormones oestrogen and progesterone, sexual stimulation and the state of the microbiotic community [21–23].

Cervical mucus is an important component of the CVF because it forms a mechanical and chemical mucosal barrier that prevents invasion of microbes and viruses. Mucus consists mostly of water (92-98%) and also contains glycoproteins, ions and antimicrobial proteins and polypeptides [11]. These glycoproteins, e.g. lactoferrin, lysozyme, immunoglobulins and defensins, have a broad-spectrum antibacterial activity [24]. The cervical gel-forming mucins play a more important role in the defence against pathogens by controlling the physical clearance of microbes. The mucins determine the amount and viscosity of the mucosal flow and therefore are in charge of the first line of defence against intruders, that washes pathogens out of the vagina [25]. It is important to note that this barrier function is not infallible: its efficiency also depends on the physicochemical [26] and microbial environment [27].

Mucins have a linear protein backbone (apomucin) that is highly O-glycosylated by oligosaccharide chains containing blood group structures. The O-linked chain starts with a N-acetylgalactosamine, α-linked with a serine or threonine, which is further extended with various monosaccharides. At the terminus, an α-linked sugar residue can be found: sialic acid, *N*-acetylgalactosamine (blood group A), or galactose (blood group B) associated with subterminal fucose (blood group O). Mucin monomers are linked together with disulfide bonds and form mucin multimers [28]. The capacity of bacteria to degrade mucins by means of microbial enzymes or mucinases, including sialidases, glycosidases, proteases, and sulphatases, is often a fundamental step in the disruption of the defensive mucosal barrier, which constitutes a direct interface between the internal and external environment of the vagina [24, 29].

2.1.2 The lactobacilli-dominated vaginal microbiome

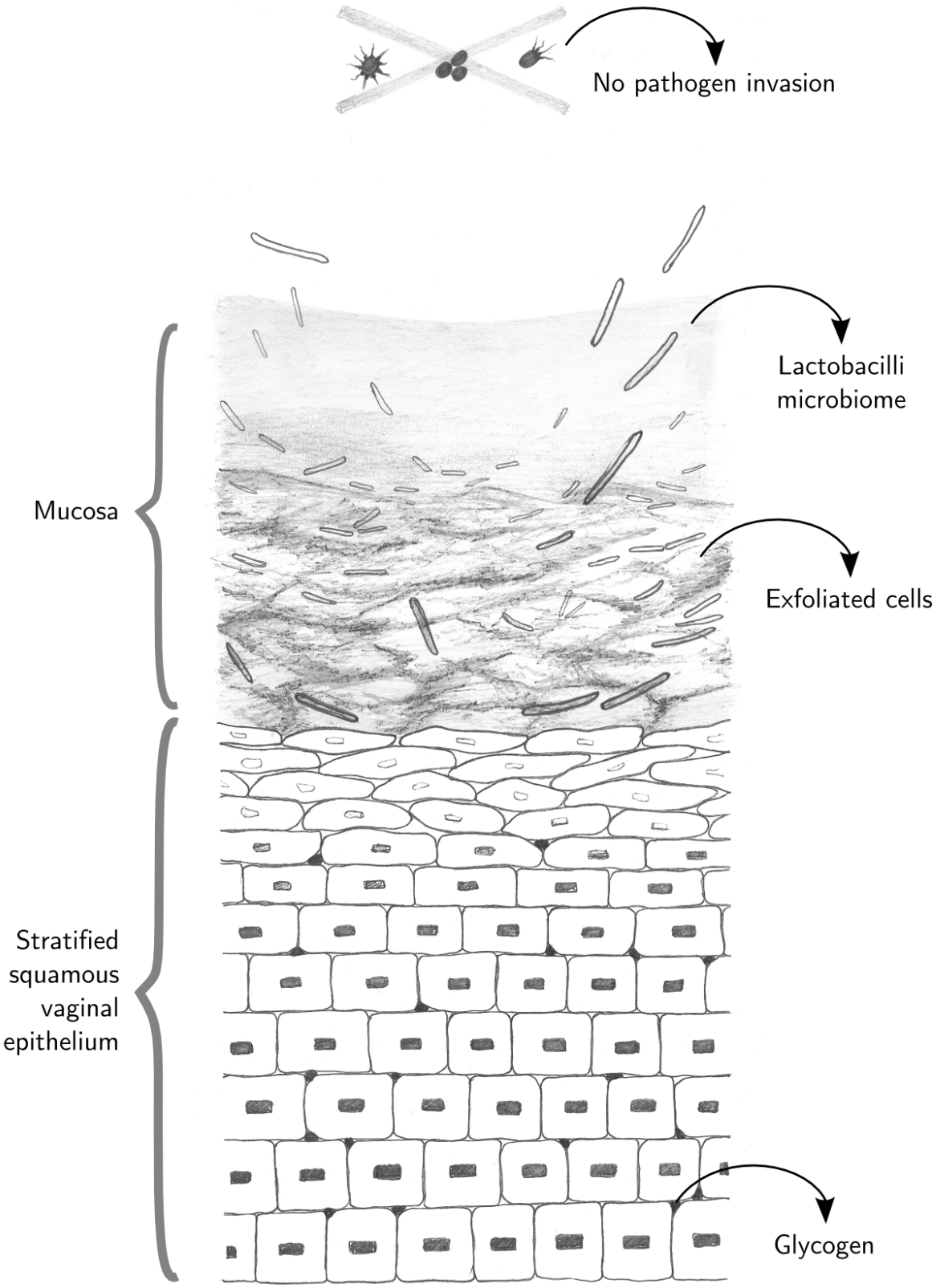
Lactobacillus species are the predominant resident bacteria of the healthy vaginal econiche. The first lactobacillus was isolated in 1894 by Döderlein from the vagina of a healthy pregnant woman [29]. The genus *Lactobacillus* comprises a phenotypically heterogeneous group of aerotolerant or anaerobic, catalase-negative, Gram-positive, non-spore-forming, rod-shaped bacteria. This genus is embedded within the lactic acid bacteria, which are functionally related through their ability to produce lactic acid [30]. A lactobacilli-dominated vaginal microbiome supports the mucosal barrier (Figure 2.2).

Both the vaginal mucosa and the *Lactobacillus* species are sources of lactic acid in vaginal secretions, which results in an acidic (pH 3.8-4.5) vaginal environment. Under the influence of oestrogen, the vaginal epithelial cells will lyse glycogen into glucose. Glucose is further metabolised into L-lactic acid via pyruvic acid using α-amylase [31]. Lactobacilli use a similar glycolysis mechanism to convert extracellular glucose into D- and L-lactic acid isomers, that have a different arrangement of the same chemical components around a central carbon atom [32–34]. The D/L ratio of lactic acid isomers found in the vaginal fluid strongly indicates that the lactobacilli are mainly responsible for vaginal acidity [33]. This low pH exerts selective antimicrobial activity against nonresident species of bacteria (and viruses and fungi) while favouring the presence of *Lactobacillus* species [23]. In addition to acidification of the vaginal fluid, the proliferation of non-advantageous bacterial species is also suppressed through *Lactobacillus'* production of broad-spectrum antimicrobial peptides (or bacteriocins) and hydrogen peroxide (whose value is still being debated), and by competing for receptor sites on the vaginal epithelium [35–37].

The most frequently isolated species of lactobacilli from the vaginal microbiome are *L. crispatus*, *L. iners*, *L. gasseri*, and *L. jensenii*. Furthermore *L. acidophilus*, *L. brevis*, *L. casei*, *L. delbrueckii*, *L. fermentum*, *L. plantarum*, *L. rhamnosus*, *L. reuteri*, *L. salivarius*, and *L. vaginalis* have frequently been isolated from women without vaginal complaints as well [3, 18, 38, 39]. *G. vaginalis* and *Prevotella* spp. are also often present in the healthy vaginal microbiome, although in relatively low concentrations [3, 15, 40, 41]. The *Lactobacillus* composition of the vaginal microbiome varies among women of geographic locations, and ethnicities.

Two species from the *Lactobacillus* genus deserve close scrutiny: *L. crispatus* and *L. iners*. *L. crispatus* is associated with a healthy vaginal microbiome and is likely to mediate vaginal protection against STIs through the mechanisms listed above [17, 27, 42–44]. *L. iners*, however, can be found both in the *Lactobacillus*-dominated vaginal microbiome and in the vaginal microbiome that is dominated by other anaerobic organisms. Moreover, in a study by Ferris et al. [45], *L. iners* was predominant in all bacterial vaginosis (BV) patients after treatment with metronidazole. On top of that, a more recent study by Petricevic

Figure 2.2: *Lactobacillus*-dominated vaginal microbiome.
The health-associated vaginal microbiome is supported by the availability of glycogen, that acts as a carbon source for *Lactobacillus* species that maintain the low vaginal pH which exerts selective antimicrobial activity. Lactobacilli also produce bacteriocins and compete for receptor sites on the vaginal epithelium with non-advantageous and pathogenic species.



et al. [46] observed an association between preterm delivery and the vaginal presence of *L. iners*, as the only *Lactobacillus*, in the first trimester of pregnancy. It has been suggested that *L. iners* is a dominant part of the vaginal microbiome at the transitional stage between health and dysbiosis [1], caused by treatment or by physiological changes. A *L. crispatus*-dominated vaginal microbiome might shift to a *L. iners*-dominated vaginal microbiome but is less likely to transition directly to a dysbiotic state [45, 47, 48].

2.1.3 Variability of the vaginal environment

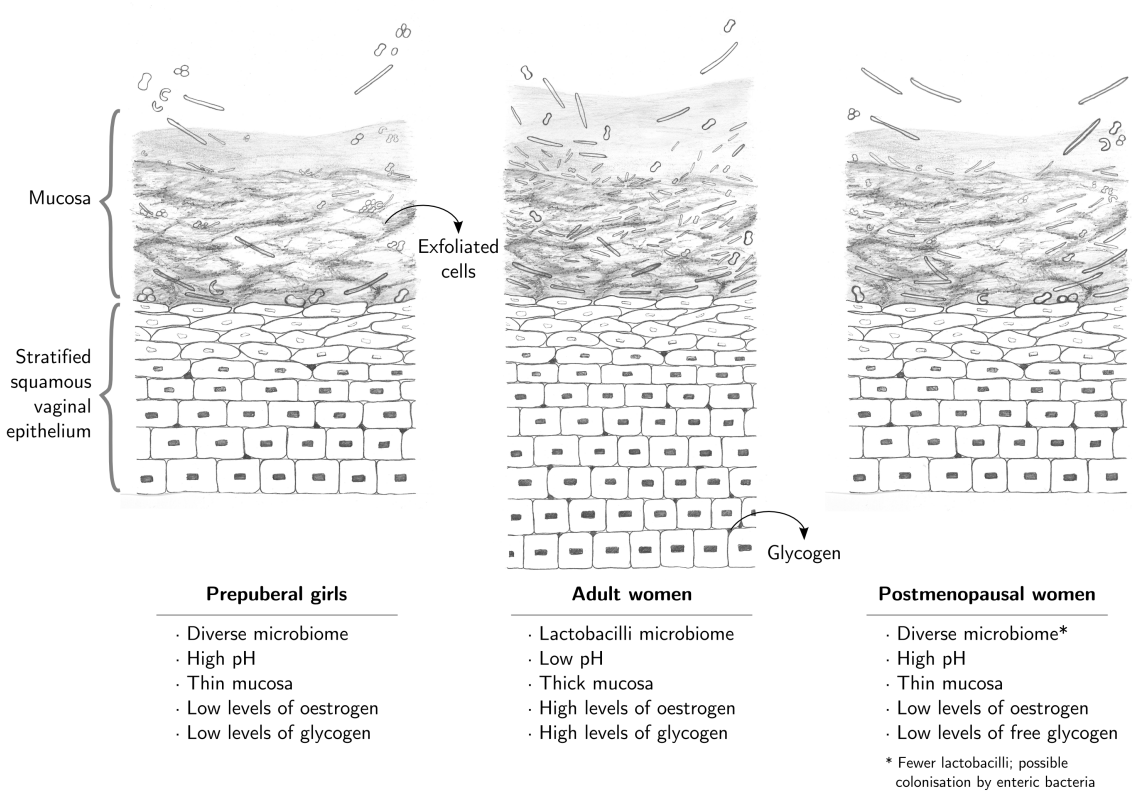
The composition of the vaginal microbiome can be influenced by exogenous factors, such as antibiotic treatment, sexual intercourse [4, 49], personal hygiene (vaginal douching) [50], cigarette smoking [51] and stress [52]. Furthermore, the vaginal microbiome is impacted by a range of endogenous factors as well. Apart from ethnicity, innate immunity, and menses, a woman's hormone levels, oestrogen in particular, have a major effect on the composition of the vaginal microbiome.

Maturation of the vaginal environment

Throughout the different stages of life, the vaginal environment is subjected to many alterations due to changes in oestrogen levels (Figure 2.3). After birth, the vaginal epithelium of the female newborn is rich in glycogen, due to the maternal oestrogen. This results in a low pH in which the maternal vaginal microbiome, that was acquired during passage through the maternal birth canal, can survive. However, shortly after birth, the decline in the maternally derived oestrogen level results in the thinning of the epithelium and the rise of the newborn's vaginal pH, in which the acidophilic bacteria no longer benefit from the selective advantage. During childhood, the vagina is predominantly colonised by a variety of anaerobic bacteria other than lactobacilli originating from the skin and the gastrointestinal tract [21, 53]. But with the onset of menarche, the increased level of oestrogen stimulates the maturing epithelial cells to release glycogen, which indirectly supplies lactobacilli with nutrients. The lactobacilli degrade glucose released from glycogen into lactic acid and again create an acidic environment, restricting the growth of pathogenic microorganisms [21, 33]. Thus, at fertile age, the normal pH of the lactobacilli-dominated vagina is 3.5 ± 0.3 [54]. After a long period of adulthood marked by a stable pH and vaginal environment, the onset of menopause and its associated decrease in free oestrogen might offer less protection from dysbiosis and possible colonisation by enteric bacteria [55–57]. In contrast, menopausal women are protected from the potentially negative effects of menses on the vaginal microbiome [15].

[1] Dysbiosis: a microbial imbalance in the body.

Figure 2.3: Stages in vaginal maturation



Menstrual cycle

The menstrual cycle is governed by hormonal changes and creates an ever-changing vaginal environment. The first half of the menstrual cycle, or follicular phase, is characterised by gradually increasing oestrogen levels which provoke an increased amount of cervical mucus that is thin and watery to allow sperm penetration. In the second half of the menstrual cycle, or the luteal phase, which is predominated by increased progesterone levels, the cervical mucus becomes scant, thick and opaque and is less penetrable to sperm [24].

During menses, there seems to be an interindividual variability, with some women maintaining a consistent vaginal microbiome, others having fluctuations timed with menses (less lactobacilli, more anaerobic bacteria) and some having random fluctuations without apparent cause [48, 58–60]. Several reports observed an overgrowth of *L. iners* can be observed during menses, while the concentration of *L. crispatus* decreases [41, 48, 59, 61].

Use of hormonal contraceptives

Hormonal contraceptives are being used by millions of women worldwide, the most widely used being oral combined (oestrogen and progestogen) contraceptives and progestin-only

injectables [62, 63]. Both contraceptive methods seem to have a protective effect on the vaginal microbiome and favour the presence of *Lactobacillus* spp. in the vaginal ecosystem [64]. The high oestrogen level and subsequent higher availability of glycogen induced by oral contraceptives probably facilitates growth of lactobacilli and subsequent lactic acid production [65]. For progestin-only injectables, this protective effect may be due to a lack of menses, but hard evidence is still lacking [65, 66].

Combined contraceptive vaginal rings (CVRs) are a common alternative to these widely used oral contraceptives. Currently, only two contraceptive rings are commercially available: the widely available NuvaRing (etonogestrel/ethinyl estradiol) and the progestogen-only Progering, only available in South-America [67]. In our research, we have focussed on the combined contraceptive ring (NuvaRing). An early clinical trial with the combined 3-ketodesogestrel/ethinyl estradiol ring could not demonstrate significant changes in the vaginal microbiome and presence of inflammatory cells, before and after use of the contraceptive ring for either 21, 28, 42, or 56 days [68]. However, another study using a combined etonogestrel/ethinyl estradiol ring (the current NuvaRing) reported a 2.7-fold increase in the concentration of H_2O_2-producing *Lactobacillus* species compared to users of oral contraception [69]. This finding was confirmed by De Seta et al. in a study in 60 volunteers that used either this contraceptive etonogestrel/ethinyl estradiol ring or combined oral contraceptives with the same steroids (desogestrel and etonogestrel/ethinyl estradiol). The ring users experienced a significant increase in vaginal lactobacilli concentration after three and six months of use [70]. It has been suggested that this positive effect on the vaginal lactobacilli population is mainly due to the local availability of ethinyl estradiol, which promotes a glycogen-rich environment [71]. In a more recent study, a combined CVR containing Nesterone^{TM} and ethinyl estradiol, that was used continuously for one year did not have any effect on the prevalence of *Lactobacillus species*, but no data on the species concentration was available [72].

2.2 Bacterial vaginosis: a dysbiosis of the vaginal microbiome

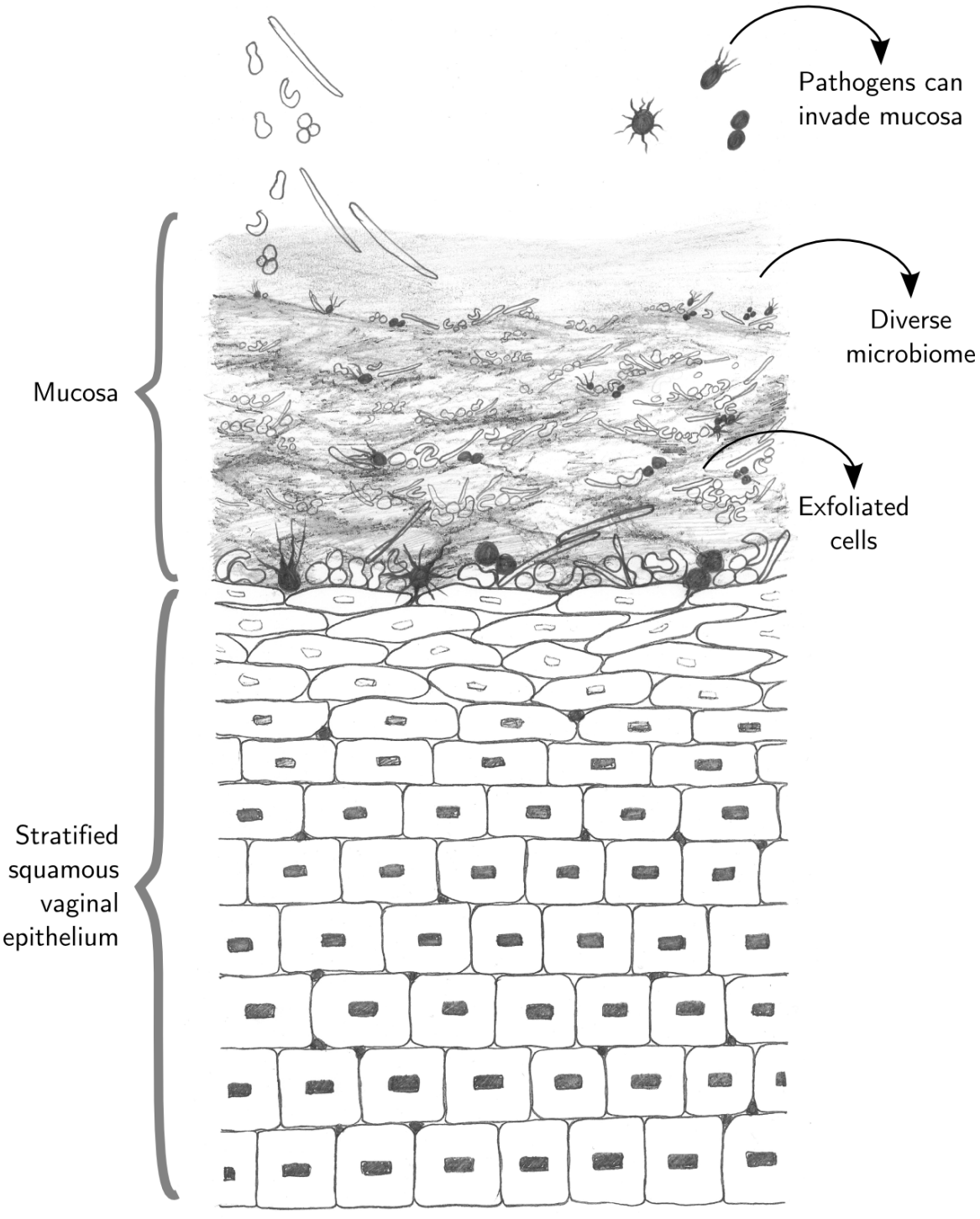
BV was defined in 1984 as follows: "A replacement of the lactobacilli of the vagina by characteristic groups of bacteria accompanied by changed properties of the vaginal fluid" [73] (Figure 2.4). The condition is characterised by a change in the microbial composition of the vagina: the *Lactobacillus* spp., associated with a healthy vaginal microbiome, are outnumbered by other microaerophilic and anaerobic organisms [15, 74, 75]. The term bacterial vaginosis was recommended because "vaginitis" suggests an inflammatory reaction of the vaginal epithelium and a high level of polymorphonuclear cells in the vaginal discharge, which is usually absent [76–80]. In the preceding years, several attempts have been made to rephrase and rename these conditions, from non-specific vaginitis over vaginal bacteriosis to anaerobic vaginosis, but none of the alternatives was generally accepted [81].

Although BV generally is not associated with symptoms of inflammation, significant variations in the cervicovaginal immune response have been demonstrated [80, 82]. BV is positively associated with proinflammatory cytokines/chemokines (IL-α, IL-1β, IL-6, IL-12 (p70), and IL-8) and negatively associated with protective antimicrobial proteins SLPI (produced by epithelial and immune cells) and elafin and the chemokine IP-10, that functions as a chemoattractant for various immune cells to the site of infection [82–84]. Furthermore, the increased diversity in the vaginal bacterial population results in increasing vaginal levels of antimicrobial effectors produced by leukocytes, such as nitric oxide (NO) [85] and heat shock protein 70 (hsp70) [86]. However, BV has also been associated with a suppressed number of leukocytes, which can explain the absence of apparent symptoms of inflammation [87].

BV is the most prevalent vaginal disorder in women of reproductive age worldwide, but disproportionately afflicts women of African descent [88], and is the most common cause of vaginal complaints [89]. BV prevalences vary considerably, but are generally highest in Sub-Saharan Africa (between 20-60%) and lowest in Europe and Asia (less than 20%), although there are exceptions to this rule [90].

More than half of the women diagnosed with BV do not report symptoms, but others may have malodorous "fishy-smelling", white, thin and homogeneous discharge and can experience vaginal itching or burning feeling [91]. The change in discharge is caused by the overgrowth of non-*Lactobacillus* bacteria. These bacteria produce enzymes (e.g. sialidases, prolidases, mucinases) that cause a degradation of the cervicovaginal mucus and increased discharge. The malodour is a consequence of the production of volatile polyamines, such as putrescine and cadaverine, by the BV-associated bacteria [75, 92].

Figure 2.4: Bacterial vaginosis-associated vaginal microbiome.
In the dysbiosis-associated vaginal microbiome, the beneficial lactobacilli are outnumbered by other microaerophilic and anaerobic organisms. This is accompanied by a degradation of the mucus layer and a disruption of the barrier function that results in increased exposure to non-advantageous and pathogenic species and viral pathogens.



2.2.1 BV-associated complications

Aside from being the cause of unpleasant symptoms, BV can also generate an entire array of serious gynaecological and obstetric complications.

In pregnant women, BV has been associated with chorioamnionitis [93, 94], premature rupture of membranes [95], intra-amniotic infections [96,97], premature labour and delivery [98–104], spontaneous abortion [98, 101, 104, 105] and low birth weight [100, 106]. These adverse pregnancy outcomes have been linked to the presence of BV-associated anaerobes, although the exact mechanism of action is still not clear. High vaginal concentrations of *A. vaginae* ($>10^6$ colony forming units (cfu)/ml) and *G. vaginalis* ($>10^7$ cfu/ml) significantly increase the risk of preterm delivery, according to Menard et al. [107]. Moreover, BV-associated bacteria including *Mycoplasma hominis*, *Prevotella* spp., and *G. vaginalis* are often isolated from the chorioamnion in preterm labor [108] and can pose a risk for intra-amniotic infections [93, 109]. Moreover, both *G. vaginalis* and *Prevotella* spp. produce sialidase, an enzyme implicated in preterm birth [110–112]. In addition, BV-associated microorganisms and their toxins are capable of crossing the placenta which could lead to brain injury in foetuses and long-term neurodevelopmental disorders in children, such as hyperactivity, academic difficulties in school and severe handicaps such as cerebral palsy [2] and periventricular leucomalacia [3] [93, 113–118]. BV is also a risk factor for the development of postpartum maternal infections [101], postabortion endometritis and pelvic infection following gynaecological surgery [119–121].

BV has been associated with histological endometritis [122] and pelvic inflammatory disease (PID) in nonpregnant women. The ascent of pathogenic bacteria such as *Chlamydia trachomatis*, *Mycoplasma genitalium*, or *Neisseria gonorrhoeae* from the lower to the upper genital tract can lead to PID [123]. These pathogenic bacteria are often accompanied by BV-associated bacteria [124, 125].

The disturbed BV-associated vaginal microbiome is associated with increased incidences of STIs [6, 126, 127] and more specifically with herpes simplex virus type 2 (HSV-2) [128, 129]. BV also creates a more permissive environment for acquiring HIV [130–132]. The presence of BV-associated bacteria in the vagina directly leads to an upregulation of HIV-replication [109, 133–135]. The risk of HIV acquisition is even higher in women, as the female genital tract is twice as sensitive to HIV compared to the the male counterpart [136, 137].

[2] Cerebral palsy: a group of permanent movement disorders that appear in early childhood.

[3] Periventricular leucomalicia: a form of white-matter brain injury that is characterised by the necrosis of white matter near the lateral ventricles.

2.2.2 The bacteria involved in bacterial vaginosis

In a lactobacilli-dominated vagina, a relative low α-diversity, or within-subject diversity, is seen, while the species diversity between different subjects is higher. Moreover, little distinction can be found in the distribution of species between different vaginal sites (mid-vagina, posterior fornix and vaginal introitus) [138]. However, when the *Lactobacillus* species are being outnumbered by BV-associated species, an increased taxonomic richness can be found, with an even higher inter-subject variability. No single dominant taxon can be found in the BV microbiome; BV patients harbour a diverse array of vaginal bacteria, many of which are only present at low relative abundance [139].

The typical spectrum of microorganisms involved in BV is well-described using conventional cultivation as well as molecular methods. *G. vaginalis* and *Prevotella* spp. are consistently found in the disturbed vaginal microbiome, but they are also present in lower bacterial loads in the healthy vaginal microbiome [3, 15, 40, 41]. Other frequently found BV-associated bacteria are *A. vaginae*, bacteria species from the *Lachnospiraceae* family (including BV-associated bacterium (BVAB) 1-3) and species in the following genera: *Bacteroides*, *Clostridiales*, *Eggerthella*, *Escherichia/Shigella*, *Dialister*, *Fusobacterium*, *Gemella*, *Leptotrichia*, *Megasphaera*, *Mobiluncus*, *Mycoplasma*, *Parvimonas*, *Porphyromonas*, *Staphylococcus*, *Sneathia*, *Streptococcus*, *Ureaplasma*, and *Veillonella* [3, 15, 18, 19, 40, 41, 80, 140–153].

Although this collection of involved bacteria seems to indicate high complexity, the vaginal microbial communities are relatively "simple" at the taxonomic level, especially when compared to more diverse microbial communities such as the oral and intestinal microbiota [138]. However, the gut and mouth can also act as extravaginal reservoirs of vaginal microbiome bacteria. Lactobacilli and BV-associated bacteria are often found in the rectum [154], and lactobacilli are found in the oral cavity [4, 155]. In adolescent girls with a healthy vaginal microbiome, nearly no oral *G. vaginalis* or *A. vaginae* was detected [4], but in women who developed BV, *G. vaginalis* was consistently found in the oral cavity [156]. Furthermore, Jespers et al. [4] demonstrated that the anorectal presence of *G. vaginalis* and *A. vaginae* was significantly higher in sexually experienced adolescent girls. And this is not without consequences: Marrazzo et al. showed that women with high quantities of oral or rectal *G. vaginalis*, or rectal *Megasphaera*, *Leptotrichia*, or *Sneathia* spp., were more likely to develop clinical BV; in contrast, women who had *L. crispatus* in the rectum were more likely to maintain their healthy vaginal environment [156]. El Aila et al. also showed strong correspondence between rectal and vaginal microbes [154].

In this thesis, the main focus will be on only two bacteria out of the full array of BV-associated bacterial species. Although this might seem to simplify a complex condition, it also allows putting the following apparent important players in the spotlight. Firstly,

G. vaginalis certainly deserves close scrutiny since it is present in up to 97.5% of cases of BV [40–42, 157], and in 50% to 70% of BV-free women, although in lower abundances [40,41,158]. This finding leads to the suspicion that *G. vaginalis* actually consists of several species with distinct roles in BV pathogenesis [159–162]. The second player is *A. vaginae*, only recently discovered and still quite unknown. The involvement of *A. vaginae* in BV was only established in 2004 [163–167] but the bacterium is rarely detected in the normal vaginal microbiome [3, 168–174]. This finding leads to the suggestion that *A. vaginae* is a better marker for BV than *G. vaginalis* and thus warrants some extra attention in this thesis.

2.2.3 Diagnosis and detection of BV

From a diagnostic point of view, a dysbiosis such as BV is very different from most infectious diseases: there is no single infectious agent that causes the condition. The condition is diagnosed based on symptoms and on the abundance of a few typical BV-associated microorganisms, which implies that BV is not diagnosed in asymptomatic women. In a clinical setting, BV is generally diagnosed using microscopic evaluation of vaginal fluid, while there is a wide array of methods available and in development to detect and investigate BV in research settings.

Amsel criteria

The most widely used method for BV diagnosis in clinical practice is based on symptoms; a positive diagnosis requires that the patient has three out of the following four Amsel criteria [91]:

1. Thin, white homogenous discharge
2. Vaginal pH greater than 4.5
3. Detection of clue cells [4] in vaginal wet smear
4. Positive whiff test: presence of amine odour after addition of 10% KOH to vaginal discharge on a glass slide

Unfortunately, this method is flawed for various reasons. The evaluation of the discharge and whiff test are subjective, and could lead to misdiagnosis. The detection of clue cells in the vaginal fluid is a subjective procedure that requires a well-trained microscopist. While the measurement of the vaginal pH is not subjective or technically difficult (since there is a wide range of commercially available pH tests), the vaginal pH can be influenced by

[4] Clue cells: Squamous epithelial cells whose surfaces are heavily coated with bacteria.

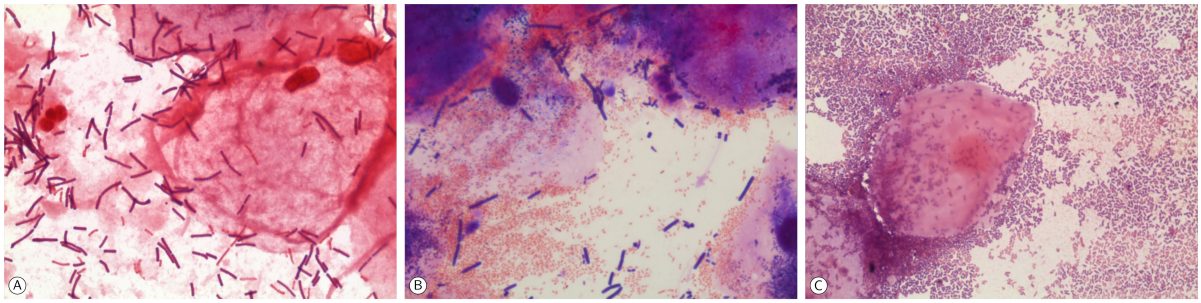
intravaginal washing, menstruation, and intercourse [55, 175] and is therefore not always reliable. Nevertheless, the Amsel criteria remain the best option for clinicians to quickly diagnose BV in their clinical practice.

Nugent score

The gold standard in BV research, the Nugent score, is a grading method that was first described by Spiegel et al. [176] and later modified by Nugent et al. [158] to include an extra category. It relies on the microscopic evaluation of Gram stained [5] smears of vaginal fluid, that divides bacteria into two groups (Gram-positive and Gram-negative) based on the properties of their cell wall. The Nugent score is based on the presence and relative amounts of three bacterial cell types (often designated as "morphotypes") in the vaginal fluid: Gram-positive rods (corresponding to lactobacilli), Gram-negative/variable pleiomorphic rods (supposed to correspond to *G. vaginalis* and *Bacteroides* species) and curved rods (supposed to correspond to *Mobiluncus* species) (Table 2.1, Figure 2.5).

The approach is used in research settings to classify vaginal smears into three categories: normal, intermediate or BV. A high abundance of Gram-positive rods, or at least the absence of *G. vaginalis* or *Mobiluncus*, gives a Nugent score of 0-3 which is considered normal. A Nugent score of 7-10 leads to the diagnosis of BV and is marked by a high abundance of *Gardnerella* or *Mobiluncus* morphotypes and the absence of Gram-positive rods. A third state between these two extreme poles is the intermediate flora, with a Nugent score of 4-6.

Figure 2.5: Nugent score.
Gram-stained smears of vaginal fluid, photographed at 100x magnification. A: Nugent 0-3 or normal state characterised by Gram-positive rods scattered around the vaginal epithelial cells; B: Nugent 4-6 or intermediate state showing a mixture or Gram-positive rods and Gram-negative pleimorphic rods; C: Nugent 7-10 or BV state with a typical dense network of Gram-negative/variable pleiomorphic rods.



[5] Gram staining: The process is done stepwise. Gram-positive bacteria retain the first dye (crystal violet) due to their thick peptidoglycan layer, and are microscopically visible as blue cells. Gram-negative bacteria lose this colouring after a decolouring step, but retain the second stain (safranin or fuchsine) and can be seen as red or pink cells.

Table 2.1: Nugent score

Score	*Lactobacillus* **morphotypes**	*Gardnerella* **and** *Bacteroides* **spp. morphotype**	**Curved Gram-variable rods**
No morphotypes present	4+	0	0
<1 morphotype present	3+	1+	1+ or 2+
1-4 morphotypes present	2+	2+	3+ or 4+
5-30 morphotypes present	1+	3+	
>30 morphotypes present	0	4+	

Total score = Lactobacilli + *Gardnerella* and *Bacteroides* spp. + curved rods

This method is less suited to the clinical setting because the Gram staining and microscopic evaluation require a certain level of technical knowledge and expertise. Furthermore, the staining is time-consuming, and it would not be feasible to perform this technique instantly when the clinician should decide on treatment of BV. But due to the greater reproducibility and objectivity, compared to the Amsel criteria, the Nugent score is still used as the standard in BV research.

There is an ongoing discussion on the designation of the correct bacterial species to these different morphotypes. Srinivasan et al. proposed, based on the results of pyrosequencing, to reclassify the pleiomorphic rods (*G. vaginalis*/*Bacteroides* species) as *G. vaginalis*, *Prevotella* spp. and *Porphyromonas* spp. and suggested that the curved rods (*Mobiluncus* species) were more likely to be BVAB1 [177]. Alternative grading systems based on Gram stained smears have also been developed. Ison and Hay have simplified the Nugent scoring system and have added a fourth category based on the presence of only Gram-positive cocci and another category to indicate that no bacteria are present [178]. This system was subsequently developed into the Claeys criteria by Verhelst et al. [179] who differentiated between the different *Lactobacillus* cell types and added a new category for the presence of diphtheroid bacilli cell types (irregular-shaped Gram-positive rods).

Commercial point-of-care tests

Several rapid diagnostics tests for BV have been commercialised and quite good sensitivities and specificities have been reported (Table 2.2). However, rapid tests have little coverage in the clinical practice, which might question the value of these reported performances.

A first type of tests is based on the symptoms of BV: increased pH and fishy odour. The self-test pH glove is based on the monitoring of pH: it was developed with a focus on pregnant women who are instructed to visit their clinician if their vaginal pH rises above 4.7 [180]. Other tests detect trimethylamine in vaginal fluid, which is responsible

Table 2.2: Performance of point-of-care tests, compared to Nugent score

Test	Test population	Sensitivity	Specificity
AromaScan [181]	n = 60 17 BV positive	94%	76.6%
Osmetech Microbial Analyser [182]	n = 372 188 BV positive	82.9%	77.3%
VGTest [183]	n = 57 18 BV positive	83%	92%
FemExam pH and Amines test card [184]	n = 219 106 BV positive	71.4%	72.8%
FemExam PIP activity test card [184]	n = 113 47 BV positive	70%	80.9%
BV Blue [185]	n = 57 8 BV positive	91.7%	98%
BV Blue [186]	n = 288 108 BV positive	88%	95%
Affirm VP III [187]	n = 176 79 BV positive	93.7%	81.4%

for the fish-like odour in BV. To detect these volatile organic amino acids in vaginal fluid, two electronic sensor assays, the "AromaScan system" [181] and the "Osmetech Microbial Analyser", [182] have been developed. The portable desktop VGtest™ ion mobility spectrometer (3QBD Ltd, Arad, Israel) also detects these malodorous biogenic amines [183]. The FemExam pH and Amines test card™ (Litmus, CA, USA) evaluates a combination of vaginal pH and the trimethylamine levels [184].

An alternative is testing the presence of enzymes in vaginal fluid. The BVBlue® (Gryphus Diagnostics, AL, USA) was developed to detect the presence of sialidase activity in vaginal fluid samples [185, 186], whereas the FemExam *G. vaginalis* PIP Activity Test Card™ (Litmus, CA, USA) detects proline aminopeptidase activity of *G. vaginalis* [184, 188, 189].

Another test based on the presence of *G. vaginalis* is the Affirm™ VP III (BD Diagnostics Systems, NJ, USA) *G. vaginalis* DNA hybridisation assay that detects high concentrations (>5 x 10^5) cfu/ml of *G. vaginalis* in vaginal fluid [187].

2.2.4 Molecular techniques for BV detection

There is a strong need to develop new and better reference methods for the detection of BV. Molecular techniques have been used since the beginning of the 21th century in research studies to detect bacteria in the human vagina, but have not yet been implemented in the clinical practice. These cultivation-independent methods have nevertheless identified a

number of novel, fastidious and uncultivable bacterial species.

Polymerase chain reaction

The first study to characterise the vaginal microbiome using broad-range polymerase chain reaction (PCR) combined with denaturing gradient gel electrophoresis (DGGE), was carried out in 2002 by Burton and Reid [190] to profile the total vaginal bacterial population. A limitation of such a broad-range method is that it tends to sample only the most prevalent bacteria and is likely to miss low-abundance or minority species [191]. Verhelst et al. [179] and Fredricks et al. [167] combined PCR with culturing of bacteria, which increases the sensitivity. The above studies have played a critical role in defining the bacteriology and identifying key organisms in BV and have paved the way for the detection of these bacteria by specific conventional or quantitative real-time PCR (qPCR). Several PCR assays using primers against the 16S-23S rRNA spacer region or 16S rRNA were developed for detection of vaginal bacteria that represent either the normal vaginal microbiome (lactobacilli), or are characteristic for BV (e.g. *G. vaginalis* [192] and *A. vaginae* [163, 164, 172]). Due to the polybacterial nature of BV, PCR [40, 170] and qPCR [3, 59, 168, 193, 194] assays for detection of a panel of key vaginal bacteria have been developed.

The use of qPCR as a diagnostic tool has been studied by several groups [45, 168, 170–174, 193, 195], but it remains difficult to apply in a clinical setting due to the expensive equipment and long turnaround time. Hence this technique is currently still better suited to a research setting. Another hindrance to the use of qPCR as a diagnostic tool is that it requires a preselection of the expected organisms. Since the aetiology of BV is still unknown and there is still some uncertainness about the relative importance of the different players, one might miss an important bacterial species when only focussing on one or a set of specific bacteria with qPCR. Jespers et al. [174] proposed a DNA tool based on log-transformed counts of the bacterial cells of *G. vaginalis*, *A. vaginae* and the *Lactobacillus* genus for the detection of BV in a research environment, which also could possibly lead to a next-generation point-of-care test for BV.

Microarray

Simultaneously measuring the expression of a large amount of genes can be achieved using a DNA microarray. A microarray is a multiplex lab-on-a-chip, in which a selection of genomic sequences (or probes) are spotted onto a solid substrate. After hybridisation of the sample onto the microarray, the relative abundance of nucleic acid sequences in the sample can be determined. A phylogenetic microarray targeting the 16S rDNA or 16S rRNA could be useful to assess the relative presence of multiple bacteria in the vaginal

microbiome in a semi-quantitative manner. An additional advantage is the fact that the composition of the microarray can be modified according to needs (for example for use in different ethnic groups). Moreover, it is possible to select multiple genomic sequences for each bacterium represented in the microarray.

A few groups have been working on the development of a tailored microarray platform which would be used as a fast, low-cost diagnostic device [17,196–199]. However, like qPCR, the technique is handicapped by the inability to detect unknown species that were not included in the set-up and, unlike qPCR, it is not fully quantitative. The technology often also requires a rather large sample volume (in terms of micrograms of DNA), which requires PCR-based amplification and thus can introduce bias into the samples. In addition, there have been reports of cross-hybridisation between similar sequences and concerns regarding the reproducibility of microarray data [200]. Moreover, Cruciani et al. [197] reported a low efficiency of their microarray in the amplification of members of the *Bifidobacteriaceae* family, which includes *G. vaginalis*. This is a major limit of the technique, since *G. vaginalis* plays a key role in the vaginal niche.

Sequencing

With sequencing the precise order of the nucleotides of a DNA or RNA strand is determined step by step. There are different ways to sequence a sample, but in general the genomic material of a sample is broken into smaller pieces that are individually sequenced and afterwards reassembled. For most approaches an in vitro cloning step is needed to amplify the genomic material, in order to increase the sensitivity of the technique. Conventional sequencing of cultured clinical isolates may provide a framework, but is unable to truly uncover the bacterial diversity in the vaginal microbiome. Bacteria present in low abundance, that could provide important information about the genetic and functional diversity of the vaginal microbiome and that may be relevant in the pathogenesis of BV, are less likely to be detected with this technique [5, 151].

To deal with this disadvantage, next-generation sequencing (NGS) has been carried out by different groups in order to discern VMB clusters in different study populations covering different ethnicities This has been carried out by different groups to detect both known and unknown sequences without prior knowledge of the bacterial species in the sample. Because the material is directly sequenced and not dependent on user-defined sequences (compared to qPCR and microarray technologies), there are no experimental bias or microarray cross-hybridisation issues to deal with. Furthermore, a small sample volume (in terms of nanograms of material) is sufficient for NGS [200]. But despite these strengths, NGS is still a rather costly technique that requires expensive equipment and highly skilled people to prepare the samples and to analyse the massive amount of data collected. Furthermore,

notwithstanding the big amount of data collected, only a small fraction of this data is trustworthy and useful for analysis.

This technology can be used to discern vaginal microbiome clusters in different study populations covering different ethnicities. The study by Ravel et al. [18] has served as the reference of a large-scale clustering study of the bacterial communities of asymptomatic North-American women of four ethnic groups. They found four lactobacilli-dominated vaginal microbiome communities, with either *L. crispatus*, *L. gasseri*, *L. iners*, or *L. jensenii* as the main contributor, that were identified mostly in Asian and Caucasian women. A fifth vaginal microbiome group with lower proportions of lactic acid bacteria and higher proportions of strictly anaerobic organisms was overrepresented in Hispanic and African American women [18]. In addition to this, other studies using similar methods to study a variety of study populations, reported similar and additional clusters (overall between three to nine clusters were found) [15, 19, 41, 143–148, 199, 201, 202]. The majority of studies found one cluster dominated by *L. crispatus* and one by *L. iners*. In contrast, clusters dominated by *L. jensenii*, *L. gasseri* and *G. vaginalis* were found less frequently, but several clusters combining *G. vaginalis* with lactobacilli were described. Furthermore, all studies found at least one (but more often more) cluster that contained mixtures of anaerobes with or without *Lactobacillus* species. Typically, this cluster contained *L. iners* and *G. vaginalis* and a group of other anaerobes. Clusters dominated by aerobes (including *Streptococcus* spp., *Staphylococcus* spp., *Escherichia/Shigella* spp. and *Proteus* spp.) were only reported in two studies [147, 201]. The most abundant taxa (other than *Lactobacillus* spp.), present in at least 50% of the studies were [15]:

- Actinobacteria: *A. vaginae*, *Eggerthella* spp., *G. vaginalis*, *Mobiluncus* spp.
- Firmicutes: *Dialister* spp., *Gemella* spp., Lacnospiraceae (including BVAB1-3), *Megasphaera* spp., *Parvimonas* spp., *Staphylococcus* spp., *Streptococcus* spp., *Veillonella* spp.
- Fusobacteria: *Sneathia* spp., *Leptotrichia* spp.
- Proteobacteria: *Escherichia/Shigella* spp.
- Sfingobacteria: *Prevotella* spp., *Porphyromonas* spp., *Bacteroides* spp.
- Tenericutes: *Mycoplasma* spp., *Ureaplasma* spp.

Fluorescence in situ hybridisation

Another molecular technique proposed for detection of BV is fluorescence in situ hybridisation (FISH) using specific fluorescent probes against the 16S rRNA of vaginal bacteria. Hybridisation-based techniques such as FISH have been developed for use in cytogenetics,

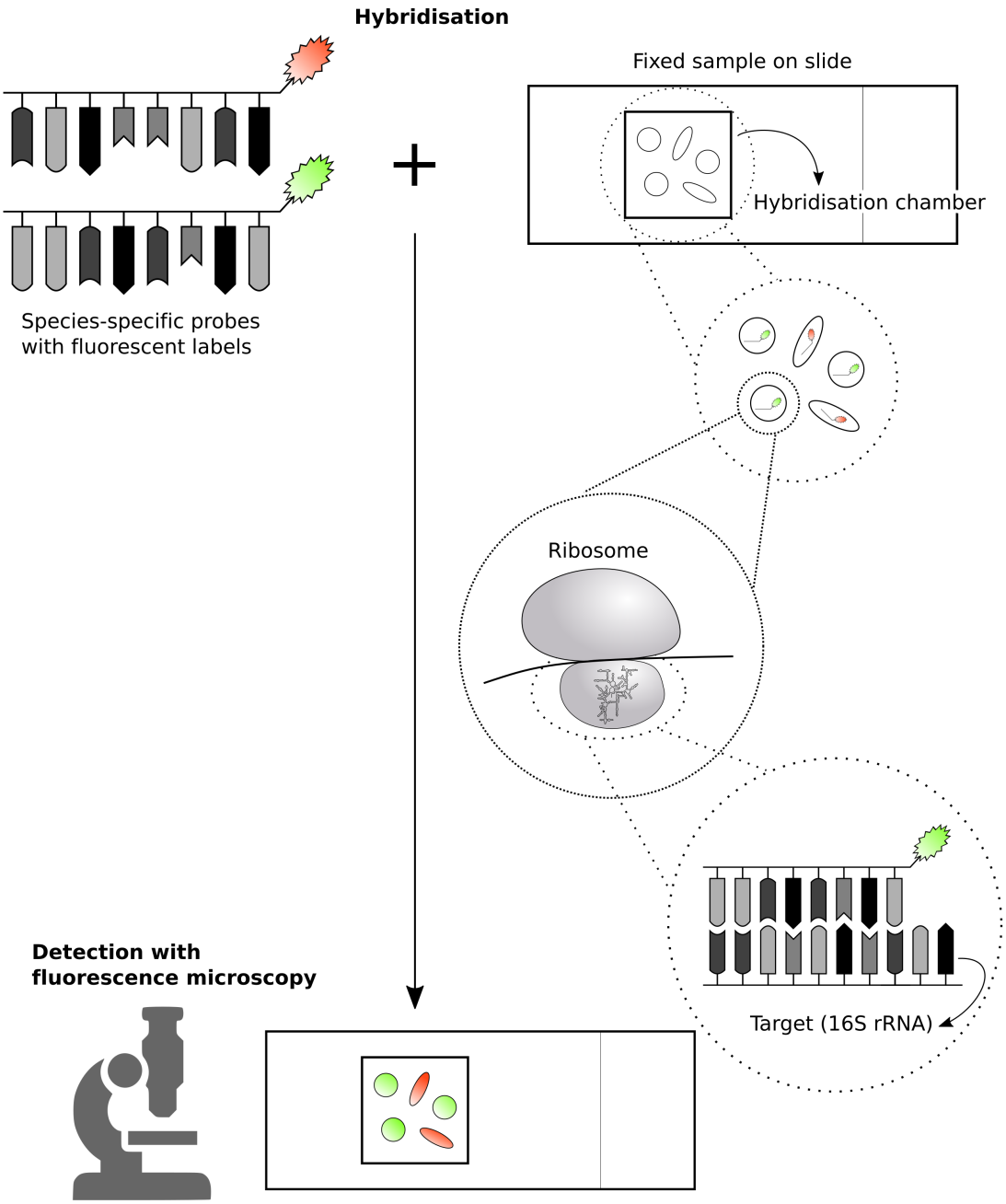
to detect the presence or absence of nucleic acid sequences on chromosomes. FISH is also being used to identify microorganisms in the field of microbial ecology, and allows visualising the distribution of a certain bacterium in a bacterial biofilm.

FISH is based on the hybridisation of a fluorescently labeled DNA or RNA sequence (or probe) with a target sequence in a biological sample and can be performed on a microscopic glass slide (Figure 2.6). Before hybridisation can occur, the double-stranded genomic sequences are denatured into single strands, using heat or chemicals. This denaturation is the result of the destruction of the hydrogen bonds between the two nucleotide strands and is needed to allow for binding the labeled probes to the complementary target sequences through new hydrogen bonds. After a wash step that stops the hybridisation reaction and removes the unbounded probes, the location of hybridised probes can be detected immediately using a fluorescence microscope.

This technique is widely used in microbial ecology and can give valuable information using FISH: the identification of microorganisms and visualisation of the distribution of specific species within a sample (for example in a biofilm). However, like most molecular techniques, FISH will only detect the user-defined bacterial species. Furthermore, similar as with other microscopic techniques, the interpretation of the FISH results can be subjective, and a well-trained eye is needed to differentiate between a genuine signal and background fluorescence. The technique can be used as a multiplex test, to detect an array of bacterial species with multiple probes at once, but it is restricted to non-overlapping spectra of fluorophores. Researchers have tried to circumvent this limitation to expand the number of distinguishable taxa in a single FISH experiment, for example by combinatorial labelling and spectral imaging (CLASI). CLASI FISH involves labelling microbes of interest with combinations of probes coupled with spectral imaging to allow the use of fluorophores with highly overlapping excitation and emission spectra in order to the simultaneously identify tens to potentially hundreds of microbial taxa in a single microscope image [203].

FISH using DNA probes was first used in BV research by Swidsinski et al. [9] for the detection of lactobacilli, *G. vaginalis* and *A. vaginae*. Fredricks et al. [167] used extra DNA FISH probes for *Mobiluncus* spp., BVAB-1, BVAB-2 and BVAB-3 to analyse the vaginal microbiome. To improve FISH efficiency, Machado et al. [204, 205] started using peptide nucleic acid (PNA) probes that have significant advantages over DNA probes to detect *G. vaginalis* and lacobacilli in vaginal smears. PNA is an artificially synthesised polymer that is similar to DNA, but with a backbone composed of repeating N-(2-aminoethyl)-glycine units (linked by peptide bonds) instead of the deoxyribose sugar backbone of DNA [206] (Figure 2.7). Compared to DNA/RNA probes, PNA probes have higher binding strength, are not easily recognised by nucleases and proteases (and thus less susceptible to degradation) and they have a superior penetration through the cell wall and hydrophobic bilayer of the target organism [206, 207]. Furthermore, they have a neutral backbone (no

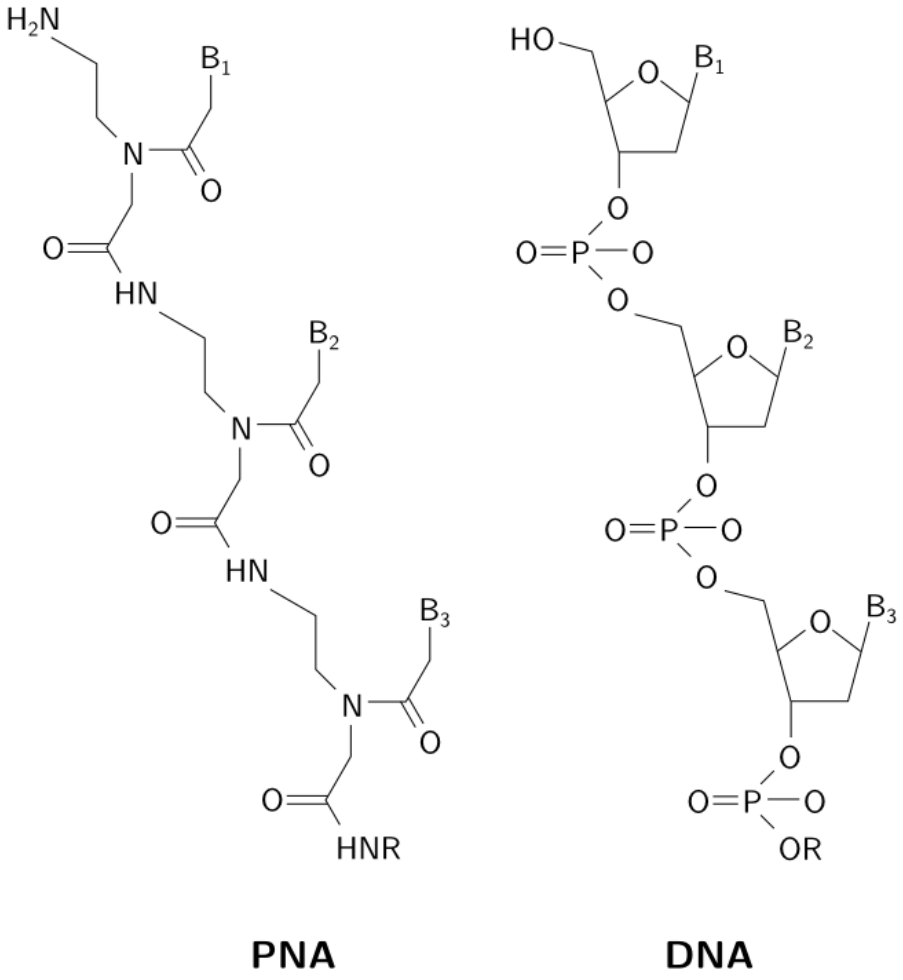
Figure 2.6: FISH technique.
A hybridisation buffer is added to the fixed biological sample on a microscopic slide, which is placed into an hybridisation oven. The buffer contains fluorescently labeled species-specific probes targeting the 16S rRNA, a component of the 30S small subunit of the prokaryotic ribosome. These labeled probes will hybridise to the complementary target sequences and the location of hybridised probes can be detected using a fluorescence microscope.



charged phosphate groups) which gives them a significant advantage in low ionic-strength conditions compared to DNA probes [208]. These low ionic-strength conditions prevent the complementary genomic sequences from reannealing when performing the FISH procedures;

they facilitate denaturation of RNA secondary structures and favour hybridisation of the PNA probes with nucleic acids [208].

Figure 2.7: Structure of PNA versus DNA.
PNA is an artificially synthesised polymer with structure that is comparable to DNA. DNA has a ribose sugar backbone, where PNA's backbone is composed of repeating N(2-aminoethyl)-glycine units linked by peptide bond. Purine and pyrimidine bases are attached to the backbone through methylene carbonyl linkages. PNA does not contain any sugar moieties or phosphate groups.



In addition to the existing PNA probes targeting the lactobacilli and *G. vaginalis*, we designed and evaluated a new specific probe for *A. vaginae* [209] (Chapter 4.2) and have used it throughout our research.

2.2.5 Models for studying bacterial vaginosis

An array of models to study BV, host-microbe and microbe-microbe interactions have already been developed, but they do not reflect the complex biological reality. Currently, no single in vitro model system is sufficient to comprise all biological, chemical and structural

human features. Different types of in vitro cell models exist, ranging from simple, relatively inexpensive models to more complex, costly systems.

- **Monolayer vaginal epithelial cell culture models** can be grown in standard tissue culture equipment using primary and immortalised vaginal epithelial cell lines. They have been used for the evaluation of epithelial immune responses and the impact and safety of products on the vaginal epithelium [210]. Bacteria can be co-cultured on monolayer cell cultures to investigate surface interactions between bacteria and cells, but bacterial growth is limited in this kind of model [211, 212].

- **Cell culture insert multilayer models** are established on insert systems. Growing cells on plastic insert with a porous membrane and exposing the upper cell layer to oxygen produces polarised, differentiated, 3D multilayer cultures. The separated apical and basal chamber facilitate studying of secretion of host products and the set-up can be used for cell migration assays. In this model, bacterial growth is also supported by carbon sources produced by the vaginal epithelium. Furthermore, the multilayer model allows for the development of biofilm, consistent with the in vivo situation. The system can also be enhanced by adding immune cells, but currently it still lacks other cell types and underlying structures that are associated with the vaginal mucosa [213, 214].

- **Rotating wall vessel bioreactor-derived 3D cell culture models** are generated when human vaginal epithelial cells are combined with collagen-coated microcarrier beads under constant low fluid shear in a fluid-filled rotating wall vessel bioreactor. The fully differentiated aggregates can be seeded into multiwell plates to study the innate immune system, epithelial barrier function (including mucus production), and epithelial-specific responses to introduced organisms. So far, no immune cells have been incorporated in this model system and it takes 28 days to culture a fully differentiated system [215, 216].

Using an in vivo animal model would be a more advanced approach to study BV pathogenesis, bacterial interactions, adverse pregnancy outcomes and the safety and efficacy of candidate products for prevention and treatment of BV. Already in 1961, Gardner and Dukes [217] unsuccessfully attempted to establish models for vaginal infection using mice, guinea pigs, rats, and rabbits. This work was succeeded by several other attempts in small-animal systems and nonhuman primates (Table 2.3). It has to be noted that the typical characteristics of the human vagina, such as the *Lactobacillus*-dominance, high availability of glycogen and lactic acid, and the low pH, are not reflected in these animal systems [210, 218]. This poses significant limitations and questions the relevance of these systems as a model for vaginal dysbiosis.

Table 2.3: Animal models used to mimic the human vaginal environment

Animal	Use	Reference
Nonhuman primates	Microbicide safety/efficacy testing	[219, 220]
	Bacterial biofilm formation on vaginal rings	[221]
	Safety of probiotics	[222]
	Vaginal colonisation with *G. vaginalis*	[223]
Grivet monkey	Model for bacterial vaginosis	[224]
Rabbit	Contraceptive safety/efficacy testing	[225]
	Foetal infection with *G. vaginalis*	[226]
Mouse	Model for group B streptococci colonisation	[227]
	Microbicide safety/efficacy testing	[220, 228]
	Model for bacterial vaginosis	[229]
	Antifungal safety/efficacy testing	[230]
	Basic research: role of mucus sialoglycans	[231]
	Inhibition of *G. vaginalis* colonisation by DNAse	[232]
	Model for non-BV and BV vagina	[233]
	Efficacy of probiotics	[234]
	Model for *Mycoplasma hominis* infection	[235]

2.2.6 Bacterial vaginosis treatment

The regimen of symptomatic treatment for non-pregnant women according to the Centers for Disease Control (CDC) in 2015 [236] was:

- Metronidazole 500 mg orally twice a day for 7 days
- *or* Metronidazole gel 0.75%, one full applicator (5 g) intravaginally, once a day for 5 days
- *or* Clindamycin cream 2%, one full applicator (5 g) intravaginally at bedtime for 7 days

Alternative regimens are:

- Tinidazole 2 g orally once daily for 2 days
- *or* Tinidazole 1 g orally once daily for 5 days
- *or* Clindamycin 300 mg orally twice daily for 7 days
- *or* Clindamycin ovules 100 mg intravaginally once at bedtime for 3 days

It is also recommended to treat all symptomatic pregnant women, using Metronidazole 500 mg orally twice daily for 7 days [236], which has been demonstrated to reduce bacterial overgrowth but not the number of preterm deliveries [237].

Unfortunately, the currently available treatments have been shown to have poor initial cure

rates in 10% to 15% of patients and relapse rates of up to 80% in those who show initial response [238, 239]. In addition, these ineffective treatments can increase drug resistance in *G. vaginalis*, *Prevotella*, *Bacteroides* and *Peptostreptococcus* spp. [240–242]. Alternatives for these failing antibiotic treatments are increasingly being explored, using probiotics, prebiotics, synbiotics, antiseptics, desinfectants, vaginal acidifying and buffering agents and combinations of different therapies, but until now, none has been successful [47, 243].

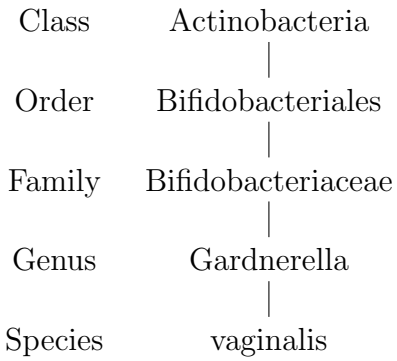
2.3 *Gardnerella vaginalis*

2.3.1 What's in a name?

G. vaginalis was isolated for the first time in 1953 from men with prostatitis and women with cervicitis by Leopold [244] and was described as a small, nonmotile, nonencapsulated, pleomorphic Gram-negative rod. It was named *Haemophilus vaginalis* by Gardner and Dukes in 1955 [245]. Later it was renamed *Corynebacterium vaginale* by Zinner and Turner [246], because it did not require hemin and nicotinamide adenine dinucleotide to grow, unlike *Haemophilus* species; because of its diphtheroid (i.e. corynebacterium-like) cell morphology; and because it had a tendency to retain violet dye after Gram staining, unlike the Gram-negative *Haemophilus* species (which implies that the *Haemophilus* species lose the crystal violet dye).

In 1980, Greenwood and Pickett [247] suggested a new genus for "*Corynebacterium vaginale*" and proposed the name *Gardnerella vaginalis*. This was supported by Piot et al. [248, 249]. Years later, Van Esbroeck et al. [250] made an attempt to place *G. vaginalis* in the Gram-positive genus *Bifidobacterium*, based on phylogenetic analysis, but despite the high level of similarity it shares with this genus, the difference in G+C content[6] between *G. vaginalis* (42 mole %) and the genus *Bifidobacterium* (55-67 mole %) is too large to consider *G. vaginalis* as a genuine *Bifidobacterium* species. To date *G. vaginalis* remains the sole member of the genus *Gardnerella*, that is part of the Bifidobacteriaceae family (Figure 2.8).

Figure 2.8: Taxonomic ranking of *G. vaginalis*



G. vaginalis was defined as a facultative anaerobic, small (0.4 by 1.0 to 1.5 µm), rod-shaped, nonencapsulated, catalase, oxidase, and β-glucosidase negative, Gram-variable[7]

[6] G+C content: the percentage of guanine and cytosine in the chromosome

[7] Gram-variable: the bacterium appears Gram-positive during the exponential growth phase, but Gram-negative as the culture ages because the thinning of the peptidoglycan layer results in an inability to retain the crystal-violet aggregates.

bacterium with a laminated cell wall, that produces acetic acid as the major end product of fermentation of carbohydrates [80, 247]. *G. vaginalis* can, due to its cell wall structure, phylogenetically be classified as a Gram-positive bacterium, even though its thin peptidoglycan layer results in the Gram-variability [157].

2.3.2 Biotypes

Piot et al. [251] assigned *G. vaginalis* to eight different biotypes, based on the activity of three enzymes: β-galactosidase, lipase (breakdown of lipids) and hippurate hydrolase. In this study, 359 strains were tested and the most common types were type 1 (β-galactosidase-positive, lipase-positive and hippurate-positive), type 2 (β-galactosidase-negative, lipase-positive and hippurate-positive) and type 5 (β-galactosidase-negative, lipase-negative and hippurate-positive). No specific differences were found in biotypes between strains isolated from women with and without BV, but up to 14% of women with BV harboured at least two different biotypes. However, a study [252] using 261 strains did find a significant difference in the distribution of biotypes from women with and without BV, with a predominance of lipase-positive strains (biotypes 1, 2, 3 and 4) in women with BV. Furthermore, women that acquired BV were likely to have a shift in biotype, suggesting that *G. vaginalis* isolates recovered from these women represented newly acquired strains rather than overgrowth of previously colonising biotypes [252]. This finding was confirmed by Numanovic et al. [253], but once again contradicted by Aroutcheva et al. [254], who found a diversity of biotypes in the BV group. In summary, the interrelationship of the different biotypes with the occurrence of BV is still controversial and unproven. A clear view is probably hampered also because several *G. vaginalis* isolates of the reference set used by Piot et al. [251] appear to be *L. iners* that were misidentified as *G. vaginalis* (Mario Vaneechoutte, personal communication).

To cope with the limitations of the phenotypic *G. vaginalis* biotyping scheme, modified techniques were implemented, albeit still based on bacterial culture [255]. More recently, molecular approaches such as random amplified polymorphic DNA (RAPD) and amplified ribosomal DNA restriction analysis (ARDRA) identified three to four different *G. vaginalis* genotypes in clinical isolates [256]. Additionally, more recent advances in next generation sequencing technology were used to differentiate *G. vaginalis* strains and subgroups according to sequence variations in 16S rRNA and the *cpn60* genes, and based on whole genome analysis [161, 162, 257, 258]. Ahmed et al. [161] suggested dividing *G. vaginalis* into four clades after the genomic analysis of 17 *G. vaginalis* isolates. Each clade was a molecular subgroup with a distinct gene pool and genomic properties, and the clades possibly correspond to four different species that differ in metabolic capabilities and virulence [159, 161]. A study by Balashov et al. [259] made an attempt using vaginal

samples to describe the four clades of *G. vaginalis* by subtyping multiplex qPCR. Clade 1 and 4 were the most prevalent clades in the vaginal specimens, and multiple clades in one specimen were found as well. The presence of clade 1, clade 3, and the presence of multiple clades correlated with BV, while the detection of clade 2 was associated with an intermediate microbiome and clade 4 did not show any correlation with BV [259].

2.3.3 Virulence factors

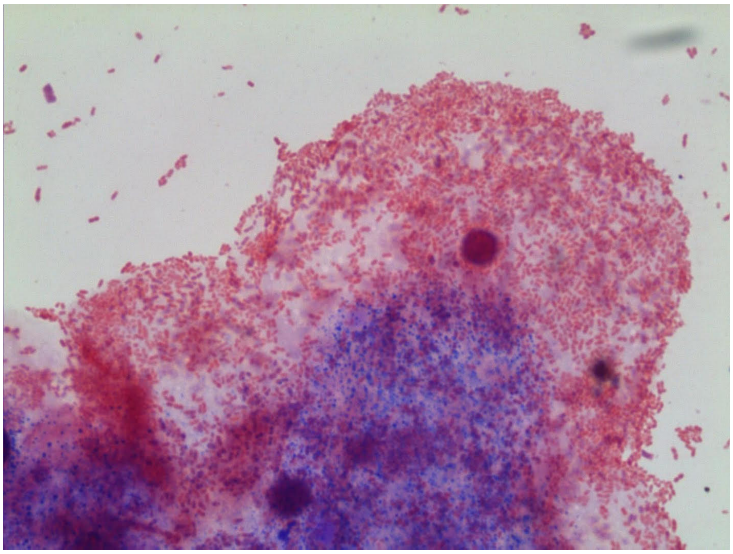
G. vaginalis is present in up to 97.5% of cases of BV [40–42, 157]. However, the presence of *G. vaginalis* in the vagina of women with Nugent scores of 0-3 indicates that its mere presence can not be used as a specific marker for BV. Indeed, *G. vaginalis* is also present in 50% to 70% of women with a Nugent score of 0-3 [40, 41, 158].

G. vaginalis is equipped with a number of virulence factors which facilitate vaginal biofilm formation and thus contribute to *G. vaginalis'* pathogenic potential. Multiple studies show that *G. vaginalis* is more virulent in vitro than many of the other bacterial species frequently isolated from BV patients [260–262]. Because of its virulence potential and its abundance in the vaginal microbiome, *G. vaginalis* is believed to be the initial coloniser of the vaginal mucosa and epithelium, serving as the scaffolding to which other bacteria can attach [9, 209, 260, 263–265]. The ability to adhere to vaginal epithelial cells has been suspected ever since Gardner and Dukes [245] postulated the value of clue cells for the diagnosis of BV. Clue cells are squamous epithelial cells whose surfaces are heavily coated with bacteria (Figure 2.9). By adhering to the vaginal cells, *G. vaginalis* could colonise the epithelium, possibly minimising contact with potentially deleterious extracellular enzymes and local antibodies, and reducing the possibility of being flushed away with vaginal fluid.

Biofilm formation

G. vaginalis can form a biofilm [9] on the vaginal epithelium, which has been suggested to increase its tolerance to lactic acid and hydrogen peroxide produced by lactobacilli [260]. The association of *G. vaginalis* in a biofilm could also explain its decreased susceptibility to antimicrobial treatment and the recurrent symptoms [266, 267]. The section on microbial biofilms will elaborate on their properties.

Figure 2.9: Clue cell after Gram stain.
Clue cells are characterised by the dotted pattern which are bacteria covering the epithelial cell.



Pili and fimbriae

A first step in vaginal colonisation is adherence to the epithelium cells, which can be facilitated by fimbriae[8] or pili. Pili[9] with a diameter between 3.0 and 7.5 nm, radiating from the surface of *G. vaginalis* cells, could be observed by electron microscopy in some *G. vaginalis* strains (but not all). Fresh isolates were more heavily piliated than laboratory strains that had been subcultured several times [223, 268].

Exopolysaccharide

Adherence to vaginal epithelial cells and clustering of *G. vaginalis* cells is facilitated by the production of an electron-dense, fibrillar extracellular polymeric substances (EPS) layer. This microcapsular material can be seen as weblike strands connecting cells lying closely together. This fibrillar layer was visualised with electron microscopy after staining with ruthenium red to visualise the polysaccharide component [269].

Iron acquisition

Next to being an essential growth factor, iron also plays an important role in the virulence of bacterial pathogens. Therefore, a number of bacteria have developed high-affinity iron acquisition mechanisms, such as secretion of high-affinity iron chelators or siderophores,

[8]Fimbria: A filamentous structure composed of proteins that extend from the surface of a cell and can be involved in attachment or can assist in disease processes

[9]Pilus: A bacterial surface structure, similar to a fimbria but longer, that is present on the cell surface in one or two copies. Type IV pili mediate twitching motility and can be involved in biofilm development

the expression of cell-surface receptors that directly bind to iron-containing compounds, and haemolysis [270]. All three mechanisms are used by *G. vaginalis*: it can produce siderophores [270] and it can bind iron carriers, e.g. catalase [271], lactoferrin [272], transferrin [272], haeme [273], and haemoglobin [274]. Additionally, by producing vaginolysin, it induces haemolysis and the release of iron-rich compounds from red blood cells [263].

Vaginolysin

The *G. vaginalis* vaginolysin is a spore-forming, cytolytic exotoxin [263]. *G. vaginalis* vaginolysin is a member of the cholesterol-dependent cytolysin family of toxins and is selective for human cells, through recognition of the complement regulatory molecule CD59 [263, 275, 276]. Vaginolysin was previously called a haemolysin because it lyses endothelial cells, neutrophils, and erythrocytes [277–281]. This ability to lyse neutrophils might explain the relative absence of neutrophils in BV [282].

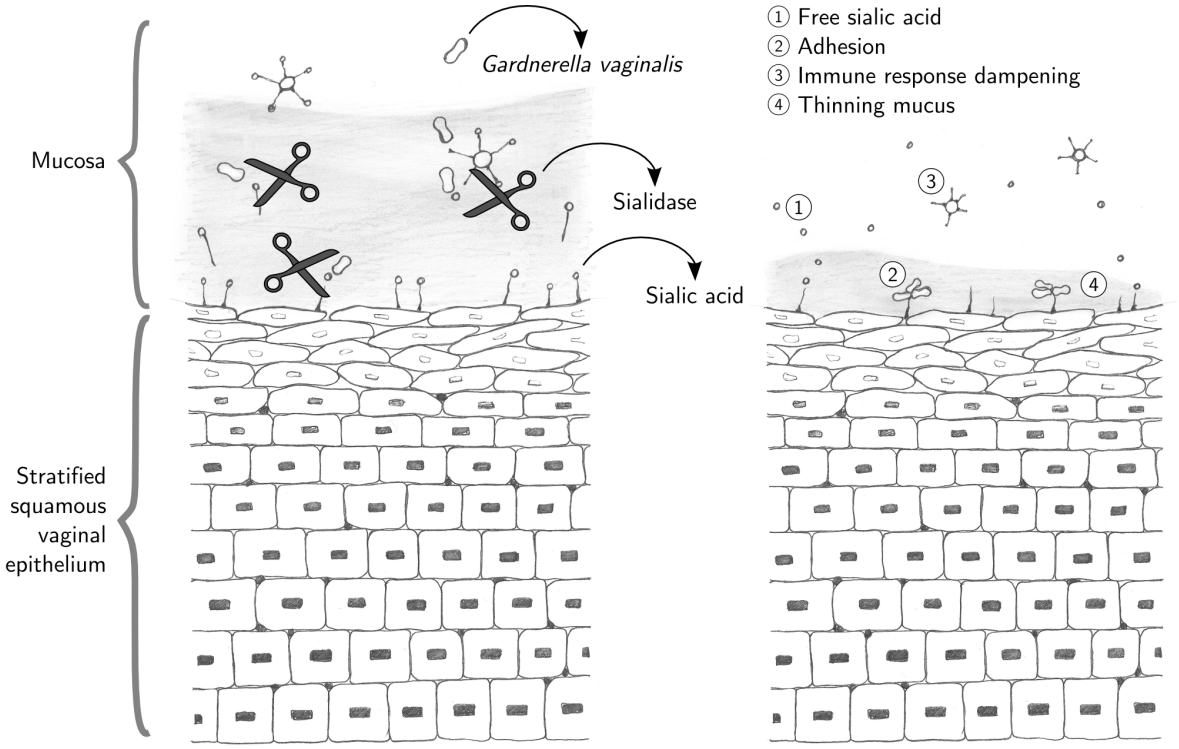
Vaginolysin can also trigger the immune system, which is upregulated in BV [82, 283]. This upregulation is mediated by the human epithelial cells inducing interleukin-8 production, and through a specific secretory immunoglobulin A (IgA) targeting the *G. vaginalis* vaginolysin [84, 281, 284].

Sialidase

Some genotypes of *G. vaginalis* can produce sialidase [160]. Sialidase, also known as neuraminidase, is a common virulence factor in various organisms such as the influenza virus [285] and a large number of bacterial species, such as *Propionibacterium acnes* [286], *Pseudomonas aeruginosa* [287], *Streptococcus pneumoniae* [288] and *Vibrio cholerae* [289] and has been strongly linked with the production of biofilm in vitro [290–292]. Increased sialidase activity was detected in the vaginal fluid of women diagnosed with BV [293]. This finding was also used in the development of a quick test for diagnosis of BV, i.e. BVBlue®, that is based on the detection of sialidase activity in vaginal fluid [294]. However, it is important to acknowledge that other BV-associated bacteria (e.g. *Prevotella* spp.) are also known to produce sialidase, hence the BVBlue® test is not specific for *G. vaginalis* sialidase.

Sialidase facilitates the destruction of the protecting mucus layer in the vagina by hydrolysis of sialic acid, which is the most distal sugar moiety on the glycans of mucous epithelial membranes [293, 295]. After cleaving off the sialic acid by sialidase, the sialic acid can serve as a nutrient [296] and the exposed glycoconjugates can serve as receptors for the bacterial cells [295]. *G. vaginalis* could benefit from this mechanism by attaching to the vaginal epithelium to initiate the formation of a biofilm. Furthermore, sialidase helps to

Figure 2.10: Bacterial use of sialic acid.
Sialidase facilitates the hydrolysis of sialic acid moieties from the mucus, resulting in thinning of the mucus layer and the availability of free sialidase which can be used by bacteria as a nutrient source. Moreover, the exposed glycoconjugates can serve as receptors for bacterial colonisation. Furthermore, by trimming sialic acid from the immune cells, the immune response is dampened, creating a more favourable environment for non-commensal bacteria.



circumvent the vaginal adaptive immune response, since it could modulate the activity of sialylated immune mediators such as interleukins, immunoglobulins and various cellular receptors (siglecs) [297] (Figure 2.10).

Cauci et al. [297] suggested that sialidase dampens the IgA response, which is triggered by vaginolysin [84, 281] by cleaving the sialic acid moieties from the IgA molecule. This results in a higher vulnerability of the IgA molecules for breakdown by proteases. This way, vaginolysin and sialidase could "team up" and increase the pathogenic potential of *G. vaginalis*. Furthermore, this process possibly renders the vaginal epithelium more permeable to HIV virions and leads to an increase in transmission of HIV [263].

Prolidase

G. vaginalis is able to produce prolidase (or proline aminopeptidase), a proteolytic enzyme that facilitates matrix remodelling and cellular infiltration. Next to sialidase, prolidase may have a role in the degradation of key mucosal protective factors (e.g. mucins, cytokines, immunoglobulins, antimicrobial molecules, and host cell receptors) and contribute to

the exfoliation and detachment of vaginal epithelial cells. It has been suggested that prolidase could modulate immune mediators causing an inefficient immune cascade after the initial IL-1β rise, which would explain why women with BV does not inflammatory signs [111, 298].

Phospholipase A_2 activity

G. vaginalis can exert phospholipase A_2 activity, which triggers the labour process [299,300]. Normal labour is initiated by amniotic and chorionic phospholipase A_2, by liberation of arachidonic acid esters from the phospholipids of these membranes, leading to the synthesis of prostaglandins by the placental membranes. In two studies, phospholipase A_2 activity from bacteria was shown to be several times higher than that of the human counterpart, and correlated with preterm labour in women with BV [299, 300].

2.3.4 Epidemiology

G. vaginalis overgrowth is found in nearly all cases of BV [40–42, 157], and has shown a high sensitivity (100%) but low specificity (49%) for BV diagnosis [80]. Nevertheless, its presence has been associated with three out of four Amsel criteria: amine odour, elevated pH, and the presence of clue cells [41].

G. vaginalis is seldom found in children, except in case of sexual abuse [301–304], although *G. vaginalis* can be found in young girls [4, 305, 306]. *G. vaginalis* is more frequently found in adolescent girls and sexually inexperienced girls, but at significantly lower rates compared to sexually active adolescents [4, 307–309]. In sexually active adult women, *G. vaginalis* is a common part of the normal vaginal microbiome [3, 15].

G. vaginalis is one of the bacterial species that can be involved in urinary tract infections (UTI), possible because of the presence of vaginal squamous epithelium in the region of the bladder trigone in postpubertal women [157,310,311]. It has been found in the rectum and in the oral cavity, which both can act as extravaginal reservoirs [4,154–156]. Carriage of *G. vaginalis* is common in men as well, and it is more often found in the genital tract than in the urinary tract [157,311]. The sole presence of *G. vaginalis* does not give rise to symptoms in most men, but the bacterium might invade the prostate or bladder [312,313] and become more pathogenic, especially in patients who have undergone a urological procedure [157]. Seminal colonisation by *G. vaginalis* also occurs [305,311,314–317] but there is no evidence that it could affect the semen quality in men [318]. Furthermore, bloodstream infections involving *G. vaginalis* occur far more frequently in women than in men, often after surgical intervention, postpartum endometritis, and septic abortion [157, 310].

2.4 *Atopobium vaginae*

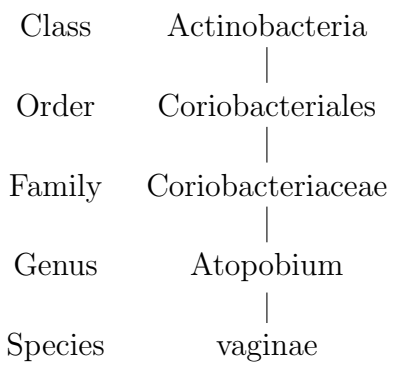
A. vaginae was only described in 1999, after being isolated from the vagina of an asymptomatic woman in Göteborg [319]. However, briefly thereafter another strain was isolated from a tuboovarian abscess [320]. The involvement of *A. vaginae* in BV was established a year later [163–167] and it has been demonstrated that *A. vaginae* is rarely detected in the normal vaginal microbiome [3, 168–174]. This finding leads to the suggestion that *A. vaginae* is a better marker for BV than *G. vaginalis*.

2.4.1 The new kid in town: the genus *Atopobium*

The genus name *Atopobium*, meaning "strange living thing" in Greek, was proposed by Collins and Walbanks [321] in 1992 to reclassify three bacterial species formerly designated *Lactobacillus minutus*, *Lactobacillus rimae* and *Streptococcus parvulus*. In 1999, *Eubacterium fossor* and a newly described species, *Atopobium vaginae*, were added to the genus [319, 322]. In 2014, a sixth species was isolated from the blood of a patient with Fournier's gangrene (in the Delta hospital in Roeselare, Belgium) and named *A. deltae* [323]. With this last addition, the genus now houses six species: *A. deltae*, *A. fossor*, *A. minutum*, *A. parvulum*, *A. rimae*, and *A. vaginae*.

The *Atopobium* species are strictly anaerobic, Gram-positive elliptical cocci or rod-shaped, nonmotile and non-spore-forming cells, and occur alone, in pairs, in clumps or in short chains. They produce major amounts of lactic acid from glucose, next to acetic acid and formic acids [319, 321]. They belong to the actinomycete branch of the Gram-positive bacteria and are sometimes confused with the low G+C-content lactic acid group of bacteria, having a G+C-content between 39%-45% (Figure 2.11).

Figure 2.11: Taxonomic ranking of *A. vaginae*



2.4.2 Epidemiology

Atopobium species occur in the oral cavity (*A. rimae, A. parvulum*), the vagina (*A. vaginae*) [322], or the pharynx of horses (*A. fossor*) [324], but can also rarely be isolated from human infections: dental infections (*A. rimae, A. parvulum*), sepsis (*A. rimae* [325], *A. deltae* [323]), abdominal wounds, and pelvic abscesses (*A. minutum*) [319].

The identification and differentiation of *Atopobium* species from other non-spore-forming Gram-positive bacilli is often laborious and carries the risk of misidentification, especially in the presence of coexisting colonising organisms, due to its variable cell morphology [80,326]. In addition, it is difficult to isolate and culture this bacterium. Therefore it is likely that the incidence of colonisation and infection of the female genital tract by *A. vaginae* was underestimated in the past.

A. vaginae has been reported to be the cause of foetal death and bacteraemia in the mother due to an intrauterine infection after a chorionic villus sample [327]. It has also been reported to be the source of intrapartum bacteraemia in a patient showing a disturbed vaginal microbiome with overgrowth of *G. vaginalis* and *C. albicans* [326].

PID is one of the possible sequelae of BV. It is a condition in which tuboovarian abscesses can be formed involving the oviduct and possibly other segments of the upper genital tract [123, 328]. PID is caused by ascending vaginal and endocervical infections. Being one of the main bacteria involved in BV, *A. vaginae* has an indirect role in PID [170, 171]. Furthermore, *A. vaginae* has also been recovered from patients with salpingitis [329] and as the sole microorganism from a patient with a tuboovarian abscess [320]. In conclusion, *A. vaginae* may be partly responsible for the association between BV and PID.

2.4.3 *A. vaginae* in BV

A. vaginae has been associated with vaginal discharge, elevated pH and the presence of clue cells, adding up to three out of four Amsel criteria [41]. The involvement of *A. vaginae* in BV has only recently been established [163, 165–167]. Since then, several researchers have demonstrated that the occurrence of BV may correspond better with the presence of high concentrations of *A. vaginae* than with high quantities of *G. vaginalis* in the vaginal tract [170, 171]. In 2005, Swidsinski et al. described for the first time the presence of a vaginal biofilm in which *G. vaginalis* and *A. vaginae* co-existed [9]. In this thesis, we elaborate on the presence of this species in vaginal biofilms in the section "Experimental work".

The involvement of *A. vaginae* in BV has a major impact on treatment of the condition. Apart from being part of a vaginal biofilm, which decreases the susceptibility to antibiotic

treatment (elaborated on in the next section), it can also be resistant to metronidazole, the standard treatment for BV. De Backer et al. [330] demonstrated that susceptibility to metronidazole varied significantly across various *A. vaginae* strains in vitro. Another in vitro study [331, 332] showed that *A. vaginae* is susceptible to clindamycin and nifuratel. Unfortunately there is a scarcity of in vivo data, but in a study with topical metronidazole gel by Ferris et al. [163], a high concentration of *A. vaginae* before treatment was associated with complete or partial failure of treatment for BV. Bradshaw et al. [170] found that rates of recurrence of BV were higher when *A. vaginae* was present in the vaginal microbiome in addition to *G. vaginalis*, even though there was a reduction in *A. vaginae* after treatment with metronidazole.

Similarly to *G. vaginalis*, *A. vaginae* can also be a strong trigger of inflammation and vaginal epithelial innate immune responses [212, 333–335]. In cervicovaginal epithelial cells in vitro, *A. vaginae* activates the major proinflammatory transcription factor NF-ϰB [212] and it significantly boosts the expression of chemokines in vaginal and cervical epithelial cells, including IL-8 [211, 212, 333, 334], MIP-3 (CCL20) [335], and RANTES (CCL5) [212, 333]. In vivo studies have shown that the detection of *A. vaginae* in vaginal specimens correlated with higher levels of the same inflammatory markers that were also associated with *G. vaginalis* [82], and that *A. vaginae* was among the most abundant taxa in the vaginal microbiome types distinguished by the highest levels of cervicovaginal inflammatory markers [336].

Since *A. vaginae* has been isolated only 17 years ago and its association with BV was demonstrated even more recently, we still know little regarding this microorganism and its relation with the commensal and pathogenic vaginal microbiome. A considerable amount of research needs to be done before denouncing this microorganism as one of the main causes of BV.

2.5 Microbial biofilm

Microbes rarely exist as single-species planktonic forms, but thrive in complex polymicrobial[10] sessile biofilm communities [337, 338].

A bacterial biofilm is a structured community of bacterial cells, adherent to an inert surface or living biological tissue and enclosed in a mucous substance ("slime"), which is a self-produced matrix of EPS [339]. This community is characterised by a complicated internal architecture: e.g. channels for circulating nutrients [340], genetically identical cells in separate areas of the biofilm that exhibit different patterns of gene expression [341] and extracellular DNA (eDNA) [232, 342, 343].

This biofilm mode of growth facilitates an enhanced tolerance to adverse conditions, which allows survival in hostile environments and offers protection against chemical disinfection, antimicrobial treatment, and human immune responses [7, 339, 344, 345]. Mixed-species biofilms are the dominant form of microbial organisation in nature, and are also prominent in the human body [7]. It has been estimated that 65% to 80% of human infections are associated with biofilm, for example *Pseudomonas aeruginosa* colonising the lungs of cystic fibrosis patients or *Staphylococcus epidermidis* infecting wounds on the skin [346].

2.5.1 Bacterial biofilm infections

The first multispecies biofilm was observed by Antonie van Leeuwenhoek more than 300 years ago, when studying "animalculi" in dental plaque, but was not recognised as such [347]. For a long time, microbial infection in the human body was envisioned as caused by single-species planktonic bacteria. This view, while explaining the pathogenesis of acute infections, was inadequate for understanding chronic and recurrent conditions [348]. To deal with the shortcoming of this classic view, Costerton et al. [339, 349, 350] investigated the association of a bacterial biofilm with these chronic infections.

Biofilm infections share clinical characteristics, regardless of the location in the human body where the biofilm develops. Although colonisation often occurs on inert surfaces, e.g. medical devices and prostheses, or on dead tissue, biofilm can also develop on living tissue, as in the case of endocarditis [339], and stagnated mucosal epithelium-associated mucus, as in the case of cystic fibrosis [7]. Biofilms grow slowly and consequently symptoms are also produced in a slow fashion [351]. Biofilm communities are rarely fully destroyed by the host defence mechanisms. Sessile bacterial cells in a biofilm release antigens resulting in an increase in antibody production. However, due to the biofilm structure, the produced antibodies are not capable of killing the biofilm bacteria and accumulate in the

[10] Polymicrobial: diverse in species and/or strain content.

surrounding tissues, which results in immune complex-related damage to these tissues [352]. Neutrophils are also attracted to the biofilm, where they continuously release antimicrobial granule contents and reactive oxygen species (ROS) that promote collagen degradation and subsequent host tissue injury as well [353]. On top of that, as antibiotic therapy fails to kill the biofilm, only symptoms caused by the planktonic cells released from the biofilm are reversed after treatment [339, 354]. As a result, even after multiple cycles of antibiotic therapy, biofilm infections continue to cause recurring symptoms [341].

2.5.2 Stages in the biofilm life cycle

Biofilm formation is facilitated by a regulated switch between the planktonic lifestyle and the sedentary multicellular state of bacteria. The biofilm life cycle includes three major steps: attachment, growth of colonies (development), and detachment of planktonic cells (Figure 2.12).

Attachment

Zobell [355] demonstrated in vitro in 1943 already that growth on a surface is more favourable to bacteria than drifting in the surrounding aqueous phase.

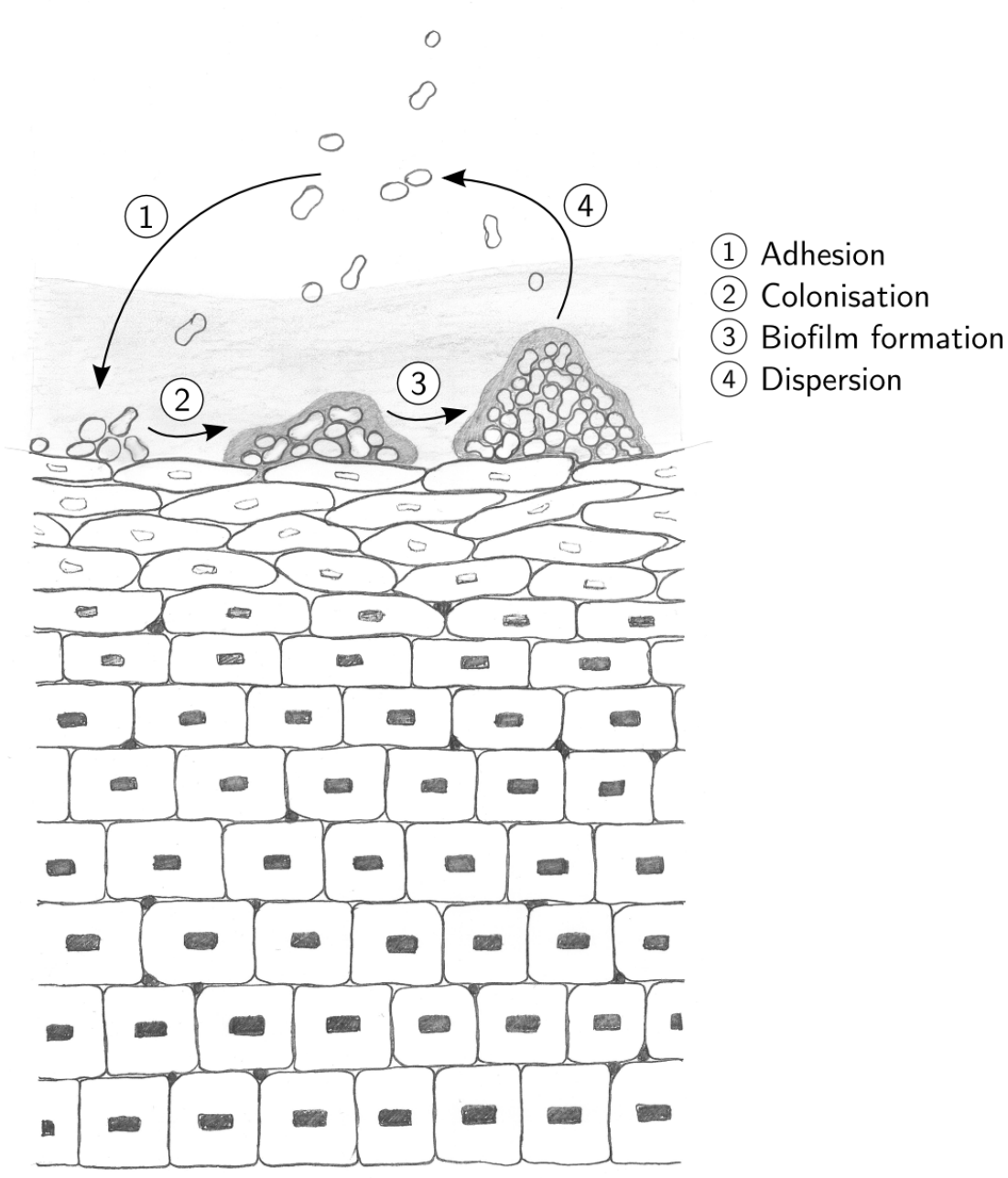
When environmental stress (such as antibiotics) favours biofilm formation, the first and most important step of the biofilm cycle is initiated. Motile and nonmotile bacterial species deploy different methods to localise a surface to colonise. In case of motile species, the individual bacterium will localise a surface and initiate a lifestyle switch, losing its motility.

Nonmotile species increase expression of adhesins on their outer surface, a mechanism that makes them "sticky" and thus promotes cell-cell and cell-surface adherence when the bacteria encounter another cell or a surface [356, 357]. Bacterial adhesion to a surface has been described as a two-phase process. The initial encounter with a surface usually leads to transient adherence, because of weak reversible bonds called van der Waals forces, that can be repulsive or attractive. If the repulsive forces are greater than the attractive forces, the bacteria will detach again [358]. However, if the bacterial colonists are not immediately disengaged from the surface, they anchor themselves permanently using their cell adhesion molecules [11] which results in a stable surface association [356, 359].

Attachment to a surface can be mediated by the bacterial capsule, fimbriae, and fibrillae through adhesins (Figure 2.13).

[11] Cell adhesion molecules: proteins on the bacterial surface that bind cells, or adhesins.

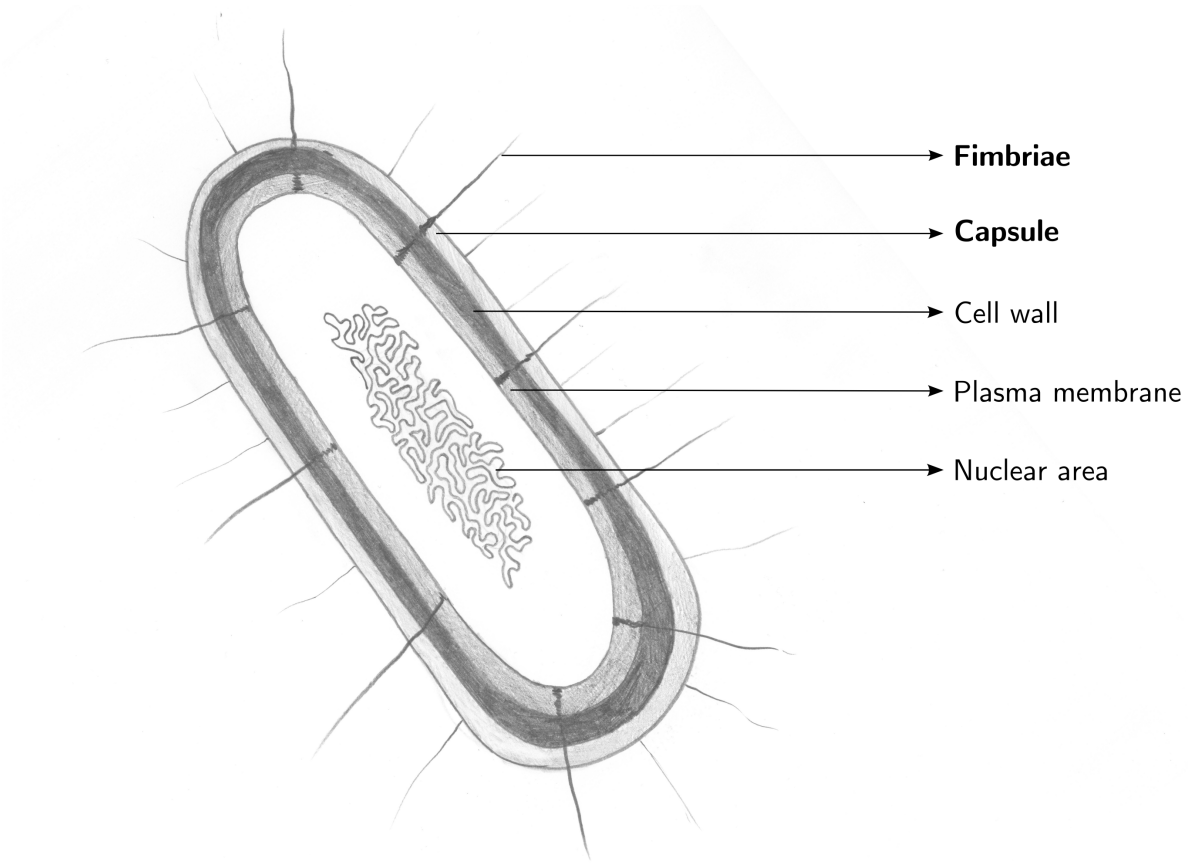
Figure 2.12: The biofilm cycle.
Biofilm is developed on surfaces, such as epithelium, and is typically formed in four stages. First, free-living bacteria adhere to the surface which leads to colonisation of this surface. After attracting more bacteria, a mature biofilm is formed when the conditions are favourable and bacteria disperse from this biofilm to return to the free-living state or to start over on another surface.



The bacterial capsule is a layer covering the bacterial cell, outside of the cell wall, can be present in both Gram-negative and Gram-positive bacteria, and is mainly composed of polysaccharides [359–362]. These polysaccharides have been suggested to act as bacterial adhesins, binding host cell surface molecules (usually carbohydrates) and could therefore be of importance in the pathogenesis of bacteria [360, 363, 364].

Fimbriae (or pili) are a group of rigid, straight, filamentous appendages on a bacterial

Figure 2.13: Mechanisms of bacterial adhesion: fimbriae and capsule.
Attachment to surfaces is facilitated by fimbriae and the bacterial capsule. Fimbriae (or pili) are a group of short, rigid, straight, filamentous appendages on a bacterial surface, prominent in Gram-negative bacteria. The bacterial capsule is a covering layer outside of the cell wall, which can be present in both Gram-negative and Gram-positive bacteria.



surface, prominent in Gram-negative bacteria and no more than 4 to 7 nm in diameter and 0.2 to 20 nm in length [359]. Fimbriae are polymers, composed primarily of identical protein subunits called pilin [365], that arise from proteins in the outer surface of the outer membrane and can be present in several hundred to one thousand copies per cell [366, 367]. Bacterial adhesion is mediated by fimbriae through associated adhesins, by adhesive subunits, or by fimbriae-dependent surface hydrophobicity [359]. Bacterial fimbriae have been shown to be related to virulence: Gram-negative bacteria possessing fimbriae are more infectious than their non-fimbriated variants [368, 369].

Fibrillae are amorphous surface adhesive structures that lack the regular filamentous forms of fimbriae, and are anchored in the cell wall. Fibrillae are more common in Gram-positive than in Gram-negative bacteria and have for example been observed at the surface of various streptococci as short, stubby appendages [359].

Several generalisations concerning bacterial adhesion and adhesins can be made [359]:

1. All bacteria can produce multiple adhesins. Bacteria can produce multiple adhesins simultaneously, to raise the probability of successful colonisation. However, they can also produce specific adhesins, appropriate for specific situations.
2. In fimbriae, adhesins are often found at the very tips of the extending fimbriae. This location, physically away from the cell surface, helps the bacterium overcome repulsive forces. Fibrillae on the other hand, are anchored in the cell wall and thus in close connection with the cell surface, making them more susceptible to these repulsive forces and therefore less capable of adhesion. "Bald" bacteria, that lack fimbriae or fibrillae, have a low tendency to adhere to surfaces.
3. A bacterium can adhere to different surfaces or cells and this ability depends on factors such as nutritional requirements, secretions, and nearest neighbours.
4. Adhesion and the resulting biofilm is the most favourable mode of bacterial growth, giving the bacteria greater resistance to enzymes, antibodies, antibiotics, and disinfectants.

Development

After this first colonisation of a surface, bacteria organise themselves into complex multicellular clusters (5-200 µm wide) [338]. This developmental progression requires multiple regulatory networks that translate signals to concerted switches in gene expression. The modified gene expression leads to spatial and temporal reorganisation of the bacterial cells within the biofilms and to observable phenotypic changes [370–373]. The biofilm grows slowly through a combination of cell division and recruitment of other bacteria and an EPS matrix is produced to envelop the biofilm bacteria. Bacteria can remain dormant on the colonised surface for a long period until the circumstances are favourable for them to overgrow, for example when the host immune function is decreased or when there is poor tissue ingrowth around a prosthesis, and this is when clinical infection occurs.

Gradients of pH, nutrients, and oxygen can be found in this microbial biofilm community. Especially the oxygen gradient can be beneficial for obligate anaerobic bacteria. Due to the consumption of oxygen by aerobic biofilm-associated bacteria, an oxygen gradient develops with an anaerobic area in the centre and an oxygen-rich environment at the surface of the biofilm. This gradient raises the possibility of multispecies organisation, in which strict anaerobes can persist in the core of the biofilm, far from the oxygen source [338, 374].

Bacterial cells in a mature biofilm are more resistant to antimicrobial treatment [359]. Hence, biofilms contain a high amount of bacterial persister cells[12] that are also shielded

[12]Persister cells: dormant variants of regular cells that form stochastically in microbial populations and are highly tolerant to antibiotics.

from the immune system because of the EPS matrix of the biofilm. These persisters play a major role in the recalcitrance of chronic infections to antibiotics [375, 376].

Detachment

When the biofilm grows in size, some cells become separated from the bulk liquid interface at the outside of the biofilm, where most essential sources of energy and nutrients are stored. In addition, waste products and toxins accumulate in the growing biofilm, and when these become trapped deep within the biofilm, they can be a threat to cell survival. To deal with this, biofilm cells have three mechanisms to escape the sessile growth mode for self-preservation and to disseminate to new locations to establish new infections: desorption, detachment, and dispersion [358, 370, 373, 377, 378].

1. **Desorption** is the transfer of bacteria directly from the substrate to the bulk liquid. This can be observed during the early stages of biofilm development when the first cell contact with the surface is initiated. Desorption is thus a passive or active reversion of the bacterial attachment process, and is likely to occur, because of the weakness of the initial bonds.

2. **Detachment** occurs when external forces, such as shear stress, become too high to maintain the biofilm structure. Bryers described in 1988 [379] four mechanisms for this detachment:

   (a) **Abrasion** is the release of cells from a biofilm as a result of collisions with particles from the bulk liquid.

   (b) **Grazing** is the removal of biofilm cells by feeding activity of eukaryotic organisms, such as amoebes, or cells, such as macrophages.

   (c) **Erosion** is the continuous loss of small biofilm portions at the biofilm-environment interface due to fluid shear.

   (d) **Sloughing** is similar to erosion, and refers to the removal of intact pieces of biofilm or the biofilm as a whole by fluid frictional forces.

3. **Dispersion** is an active release from the biofilm. It is characterised by a phenotypic switch and sensing of certain signals and cues. These cues are translated through regulatory networks that enable physiological changes to facilitate cellular release. There are two types of dispersion: first, native dispersion, also known as seeding dispersion, which occurs upon sensing of self-synthesised signalling molecules and which is usually the terminal stage in biofilm development, and second, environmentally induced dispersion, triggered by factors in the external environment (e.g. starvation,

oxidative stress, availability of oxygen and nutrients,...). Dispersion rarely involves the entire biofilm, but only selected microcolonies or areas within a biofilm.

2.5.3 The biofilm matrix

In many biofilms, the microorganisms account for less than 10% of the dry mass, whereas the matrix can account for more than 90% [343]. The biofilm matrix is the "house of biofilm cells" and determines the immediate living conditions of the bacteria in a biofilm by affecting porosity, density, water content, charge, sorption [13] properties, hydrophobicity, and mechanical stability [380, 381]. Exopolysaccharides are an important part of the extracellular matrix, that additionally comprises a range of biopolymers of microbial origin, such as proteins, glycoproteins, glycolipids and eDNA [381]. It is difficult to provide a complete biochemical profile of the biofilm matrix because of the complex consistency and the challenging carbohydrate chemical analyses.

Nevertheless, it has been established that all EPS biopolymers are highly hydrated and form a matrix that keeps the bacterial cells together, retains water and interacts with the environment [381]. Water is the most predominant component of the biofilm matrix (90-97%). The matrix is a highly hydrated environment that dries more slowly than its surroundings, which results in a buffering function against fluctuations in fluid potential [343]. The matrix provides mechanical stability to maintain the biofilm architecture during prolonged periods. It acquires this stability by establishing hydrophobic interactions, cross-linking by multivalent cations, and entanglements of the biopolymers [380, 382].

Facilitating dense living circumstances, biofilms are ideal for exchanging genetic material and maintaining a large and accessible gene pool. The close proximity of neighbouring cells promotes horizontal gene transfer, thus the exchange of genetic information, between the not fully immobilised bacteria. There is also interaction between components of the EPS matrix, for example to retain extracellular proteins [380]. This mechanism is crucial for preventing wash-out of enzymes, allows effective metabolisation and degradation of polymeric and particulate material, and leads to an "activated matrix" [343, 381]. This activated matrix, which becomes even more dynamic by the release of membrane vesicles[14]; i.e. highly ordered nanostructures that act as parcels, to be sent deep into the EPS matrix, for delivery of enzymes to strengthen the biofilm and the release of nucleic acids to enhance gene exchange [383].

[13] Sorption: a physical and chemical process by which one substance becomes attached to another by absorption, adsorption, or ion exchange.

[14] Membrane vesicle: a vesicle that is formed from the outer membrane of Gram-negative bacteria and is secreted from the cell surface.

Multi-species biofilms

Most bacteria live within a multi-species biofilm, and they also interact with their neighbours. These interactions can be antagonistic or synergistic and include communication via quorum sensing, and metabolic cooperation or competition. Given that mixed biofilms are ubiquitous, synergetic interactions seem to dominate over antagonistic ones, and this can result in several beneficial phenotypes. For example, bacteria can have a metabolic collaboration where one species utilises a metabolite that was produced by a neighbouring species [7]. Another example of bacterial cooperation is the process where one of the members of the biofilm provides better living conditions for another member. This is the case for anaerobic bacteria that are sensitive to oxygen, but are able to survive and persist under aerobic conditions in the presence of aerobic bacteria. The aerobic members of the biofilm consume the oxygen and provide anaerobic conditions within the deeper layers of this biofilm, allowing the anaerobic members to survive and multiply [374, 384].

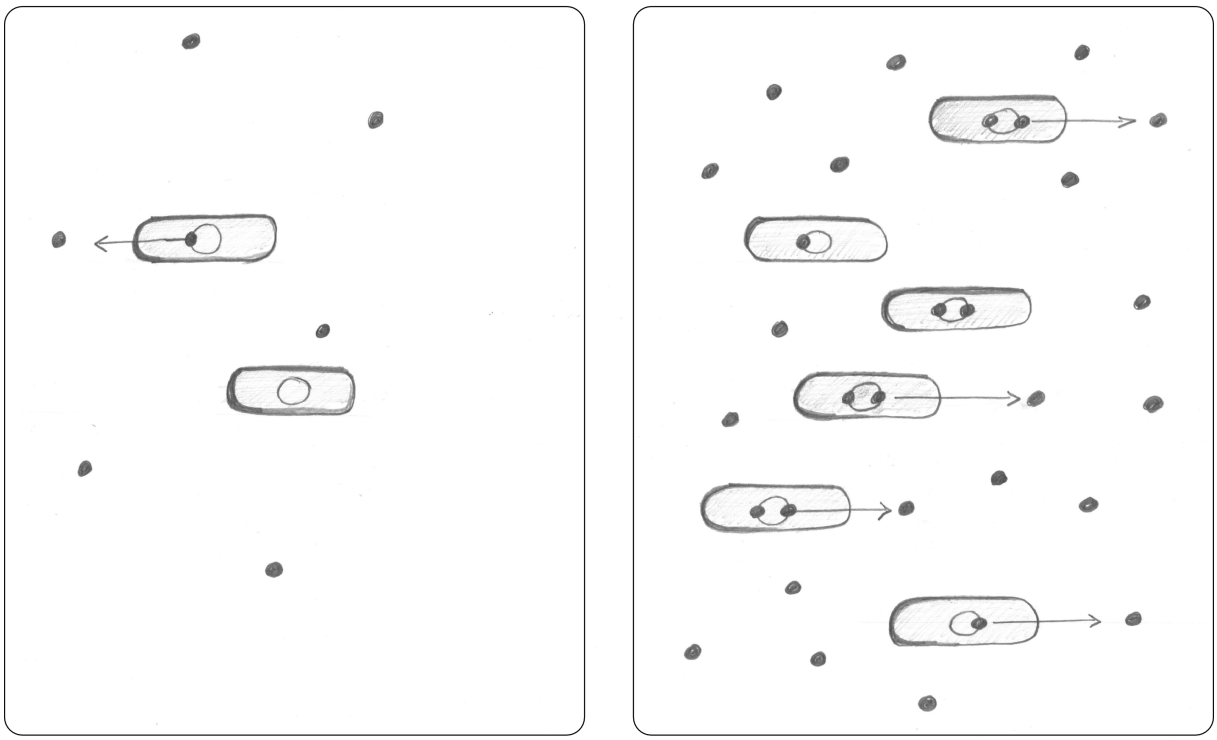
2.5.4 Communication between microbial cells

Communication between neighbouring bacterial cells occurs by quorum sensing. It allows bacteria to monitor the environment for other bacteria and to alter their behaviour in response to changes in the number and/or species present in the community. It enables bacteria to act as multicellular organisms [385]. Quorum sensing facilitates interactions within bacterial communities and is used to initiate colonisation and aggregation, and to establish a biofilm. The communication process requires the production and release of small hormone-like chemical signalling molecules, called autoinducers. These autoinducers can increase in concentration as a function of cell density or dependent on physiological conditions, which makes quorum sensing only beneficial when it is carried out simultaneously by a large number of bacteria and when a positive feedback loop causes the entire population to switch into "quorum sensing mode" [385, 386] (Figure 2.14).

Several quorum sensing systems have been described. While some are mainly used for intraspecies communication, others support interspecies communication and enable bacteria to sense the presence of other bacterial species. The universal autoinducer-2 system has been identified in several Gram-negative and Gram-positive bacterial species and can mediate interspecies communication [385, 387]. The system was discovered in *Aliivibrio fischeri*, a bacterium that lives in symbiosis with marine animals such as the bobtail squid [388]. It is used by a wide range of bacterial species, such as human oral commensal bacteria that use the autoinducer-2 system for mixed biofilm formation and development of dental plaque [7].

Due the spatial heterogeneity and biodiversity in mixed-species biofilms, the "calling dis-

Figure 2.14: Communication between microbes: quorum sensing.
Bacteria produce small, diffusible signal molecules, that accumulate in confined surroundings when the bacterial population increases until it reaches a threshold. When the threshold is exceeded, there will be concerted gene modulation throughout the population which can result in biofilm formation, for example. Quorum sensing allows bacteria to switch between two distinct gene expression programs: one that is favoured at low cell density for individual behaviour, and another that is favoured at high cell density for group behaviour.



Low bacterial density: individual behaviour

High bacterial density: group behaviour

tance" can be an important limitation in quorum sensing. Egland et al. [389] demonstrated that signalling occurred mainly within cell clusters, rather than across them. Therefore it is suggested that the distance between bacteria may be more important than the amount of cells present in the environment. Sufficient accumulation of autoinducers is thus regulated by cell aggregation rather than by population density [390].

2.6 Treatment of biofilm infections

2.6.1 Resistance versus tolerance

Resistance is the act of fighting against something that is attacking you. Antibiotic resistance is the resistance of a bacterium to an antibiotic used for treatment or prevention of an infection caused by that bacterium. Bacteria can be naturally resistant, or can acquire resistance through genetic mutation, or by gene transfer from another bacterium. These acquired genetic mutations often involve familiar mechanisms of resistance, such as efflux pumps, modifying enzymes, and target mutations [8].

However, it has been demonstrated that bacteria living in a biofilm are not per se resistant to antibiotics, but have a higher tolerance towards antimicrobial treatment [8, 267, 375]. Tolerance is being defined as the organism's ability not to be harmed by a drug over a continuous period of time. As such, it is a coping strategy against antimicrobial treatment.

2.6.2 Increased tolerance towards antibiotics in biofilm cells

Living in a biofilm results in increased tolerance of bacterial cells towards antibiotic treatment. Biofilm-associated bacteria can become 10 to 1000 times less susceptible to antibiotics compared to planktonic cells [391, 392]. There are multiple reasons for this, including decreased and slower penetration of relatively large antibiotic compounds through the viscous matrix. The matrix components can also chemically neutralise antimicrobial compounds [393]. However, the biofilm matrix does not form an entirely impermeable barrier for all antibiotics, as demonstrated by mathematical models [394], so there must be other mechanisms that play a role. Bacteria can develop an increased tolerance towards antibiotics without undergoing genetic changes, and these bacterial cells are known as persisters. The first description of this phenotype has been done by Hobby et al. in 1942 [395]: they discovered that 1% of *Staphylococcus aureus* cells were not killed by penicillin. Persister cells usually comprise about 1% of biofilm cell, are in a state of dormancy, and exhibit a reduced growth rate and a decreased metabolism [396]. This implies that the persister cells do not undergo cellular activities, which can be corrupted by antibiotics [396].

Moreover, persister cells can also lead to relapses after treatment. The biofilm contains both regular and persister cells which are occasionally shed off into the surrounding tissue and bloodstream. Shedded regular cells are killed by the antibiotics and the immune system is able to eliminate shedded persister cells. In the meanwhile, persister cells in the core of the biofilm survive because they are shielded from the immune system by the

matrix. When the concentration of antibiotic compounds drops, the persister cells can repopulate the biofilm, causing a relapse in infection [376].

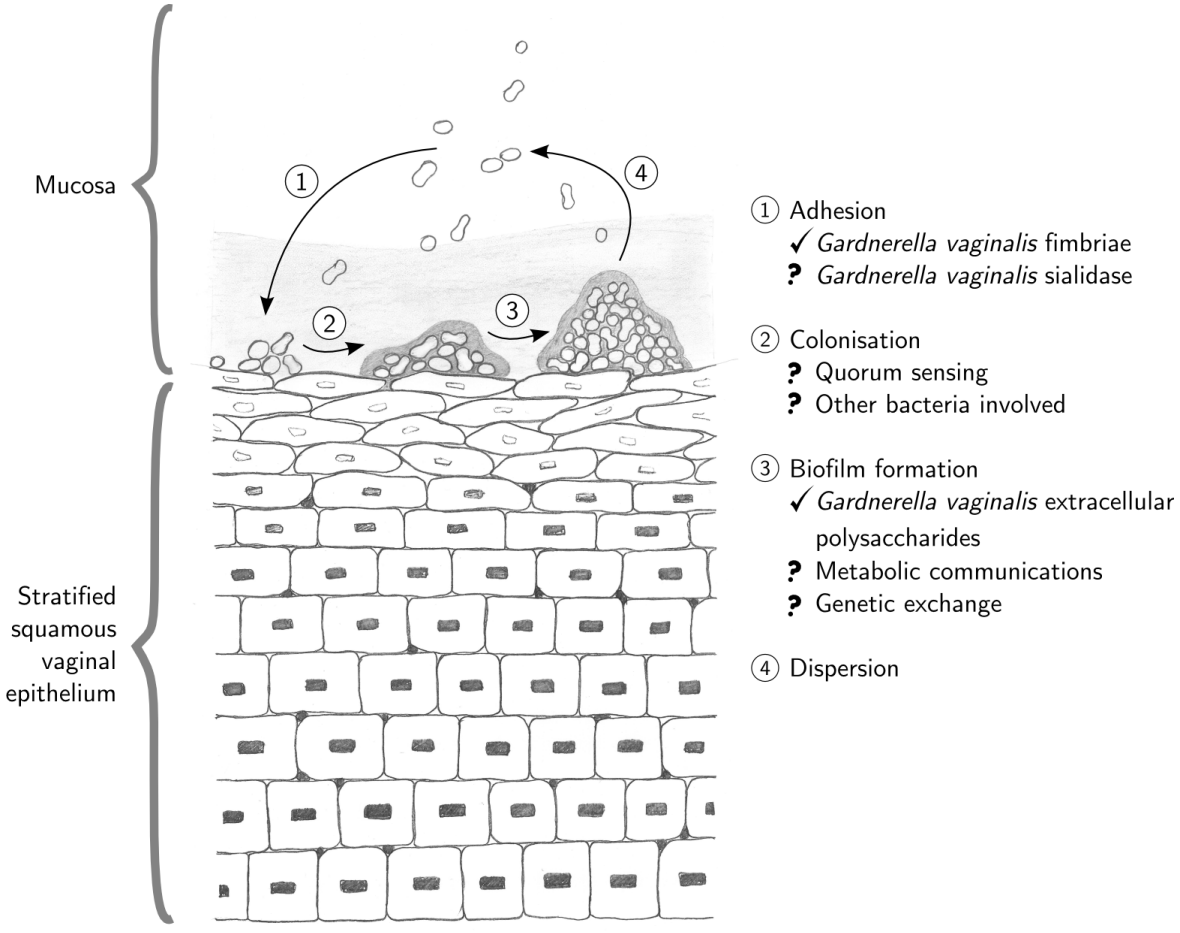
2.6.3 New treatment strategies for biofilms

Treating a biofilm infection is challenging, due to biofilm bacteria's intrinsic resistance, or due to increased tolerance towards the available antimicrobials and the innate immune system. Therefore, new targets for therapy and prevention of biofilm infections have been studied [375]. Prevention strategies include manipulation of abiotic and biotic surfaces with anti-biofilm components [397] and external stimulation of the innate immune response [398, 399]. New treatments could be targeted at diminishing the biofilm buildup or damaging the established biofilm (e.g. matrix, persister cells), which could be done by interfering with quorum sensing signalling [400]. Another promising approach results from the research on pilicides and curlicides, novel compounds currently being developed against *Escherichia coli*'s fimbriae that are involved in biofilm formation [401]. Other anti-biofilm agents that are being studied are inspired by the human immune system: for example the cationic human host defence peptide LL-37, suppressing stress responses [402] or nitric oxide promoting biofilm dispersal through intracellular secondary messenger cyclic di-GMP [403, 404].

2.7 Biofilm in bacterial vaginosis

The ability of *G. vaginalis*, the most representative bacteria in BV, to colonise human cells has already been established in the eighties [223, 405]. However, it was not until 2005 that Swidsinski et al. [9] used FISH to demonstrate the presence of a polymicrobial biofilm adhering to the vaginal epithelial cells in BV. After this first visualisation, other researchers have developed probes for other associated bacteria to visualise microorganisms involved in BV [204, 209, 406]. The presence of epithelial cells covered with bacteria, or clue cells, is one of the Amsel criteria used in clinical settings to diagnose the condition. Such coating of epithelial cells with multiple layers of bacteria is exactly what one expects to see in case of biofilm formation.

Figure 2.15: Biofilm in bacterial vaginosis: what we knew before



In reality, we have been looking at clue cells for decades, without realising that we were dealing with biofilm formation. Furthermore, BV treatment is very challenging, due to recurrence and relapses after antibiotic therapy, as is the case in other biofilm-associated infections. Little is known about the exact mechanisms of biofilm formation in BV: the genes responsible, the communication strategies (quorum sensing, metabolic

communication), and the genetic exchanges between the biofilm-associated bacteria (Figure 2.15). Furthermore, although it has been established that BV is a polymicrobial condition, which involves a polymicrobial biofilm, currently we do not know the exact bacterial composition of this biofilm and the importance of the separate members.

We do know that *G. vaginalis* is an important player in BV, even though it is also prevalent in the healthy vaginal microbiome. Moreover, *G. vaginalis* has multiple virulence factors that may contribute to the development of a biofilm. Specifically, the presence of fimbriae [157] and the ability to produce sialidase [111, 160] play a major role in the colonisation of the vaginal epithelial cells, and its potential to produce EPS [157] could be important in the maturation of the biofilm. It is therefore very convincing that *G. vaginalis* is the initial coloniser that functions as the scaffolding to which other bacteria attach in order to establish a mature biofilm and benefit from the synergy between the different members of the biofilm. One of these secondary colonisers could be *A. vaginae*, an obligate anaerobic bacteria, that more recently has been associated with BV. One reason for the proliferation of *A. vaginae* may be the presence of an oxygen gradient within the biofilm. By embedding itself within the biofilm, *A. vaginae* can take advantage of the anaerobe environment and proliferate in a mutualistic relationship with *G. vaginalis*.

Microorganisms are able to attach to the surface of indwelling medical devices, and cover these surfaces with biomass [407, 408]. Currently little information is available on whether a vaginal device, such as a contraceptive ring, influences vaginal biofilm formation, and on whether the dysbiosis state of the vaginal microbiome could result in a denser biomass on the CVR. At this moment, only two studies have investigated biofilm formation on the contraceptive NuvaRing. Miller et al. [409] examined a NuvaRing after four weeks of use by one healthy volunteer and observed only cellular debris, but no bacterial growth on the surface of the ring using scanning electron microscopy (although at a very low magnification). Another study demonstrated biofilm formation on the NuvaRing by *Candida albicans* and *C. tropicalis* in vitro [410]. Studies using other types of vaginal rings in women and female pig-tailed macaques showed evidence of biomass formation on the rings using microscopic techniques [221, 411]. More research on this topic is urgently needed, since there is an ongoing trend to incorporate products for BV prevention (lactic acid, probiotics) in future rings.

In this thesis, we study *G. vaginalis* as one of the main initiators of a vaginal biofilm, and investigate the role of *G. vaginalis'* sialidase gene as a virulence factor for the development of this vaginal biofilm (Chapter 4.4). We further investigate the association of *G. vaginalis* with *A. vaginae*, a second relevant bacterial species in BV (Chapter 4.2 and 4.3). In addition, we assess the association between the state of the vaginal microbiome and the colonisation of CVRs and describe the composition of this CVR biomass (Chapter 4.5).

CHAPTER 3

Rationale and objectives

Despite the 10 years of research since the first description of a biofilm in bacterial vaginosis (BV), still little is known about the role of the BV-associated bacteria in the process of biofilm development. *G. vaginalis* has been proposed as the main initiator of BV development by establishing the first attachment to the vaginal epithelium and thus acting as the base of the biofilm [141, 264]. This hypothesis still lacks hard evidence and various researchers are looking into this mechanism. The study leading to this thesis also aspired to unravel this process of biofilm development in women with BV.

The specific objectives of this thesis are:

1. To further characterise the biofilm associated with BV.
2. To identify, quantify and visualise the microorganisms involved in the BV biofilm, and more specifically *G. vaginalis* and *A. vaginae*, using qPCR and FISH.
3. To study the association of *A. vaginae* with *G. vaginalis* in the biofilm.
4. To research the association between the presence of the sialidase gene of *G. vaginalis* and the development of a biofilm on the vaginal epithelium.
5. To study the effect of vaginal dysbiosis and vaginal biofilm on vaginal contraceptive rings (CVRs).

The next chapter of this thesis, representing four different research papers, deals with these questions.

Unraveling the bacterial vaginosis biofilm: technical preparation To initiate the characterisation of the biofilm in BV, we looked for appropriate tools. We opted to use FISH to visualise bacteria of interest using microscope slides with vaginal fluid. After testing and validating existing FISH probes, we established that the available probe for *A. vaginae* was not specific enough and we decided to design a new, specific one. Chapter 4.2 describes the design and validation of the new AtoITM1 probe. FISH was employed using this new probe together with an existing probe for *G. vaginalis* and a broad-spectrum bacterial probe as positive control.

A fruitful alliance: the synergy between *A. vaginae* and *G. vaginalis* In chapter 4.3 we demonstrate our newly set-up technique on vaginal specimens collected for the Ring Plus study. The Ring Plus study was set up to study the safety and acceptability of a CVR, used by 120 women for a period of three months. We studied the possible synergy between *G. vaginalis*, which is present in the healthy vaginal microbiome in addition to the BV microbiome, and *A. vaginae*, a bacterium that may be more specific for the diagnosis of BV.

Sialidase-producing versus non-sialidase-producing *G. vaginalis* in biofilm Because *G. vaginalis* is also present in the healthy microbiome, we investigated differences between different types of *G. vaginalis*. One possibly important factor is the production of sialidase, which may facilitate adhesion to the vaginal epithelial cells and thus the first step in biofilm formation. In chapter 4.4 we assess the association between the presence of a sialidase-producing gene in *G. vaginalis* and the occurrence of BV-associated biofilm of *G. vaginalis* on the vaginal epithelium.

The impact of the vaginal biofilm on intravaginal rings Microbes are known to adhere to all surfaces and especially to inert surfaces of medical devices that are brought into the human body. Nowadays more and more research is being done on the use of intravaginal rings (IVR) for contraception, and for prevention and treatment of STIs, but little data are available on the effect of an IVR on the vaginal microbiome and vice versa. Chapter 4.5 deals with the association between the vaginal microbiome state and the buildup of biomass on CVR.

CHAPTER 4

Experimental work

4.1 The Ring Plus study

The data used in this chapter was obtained using vaginal samples of the Ring Plus study, a multidisciplinary research project at the Rinda Ubuzima in Kigali, Rwanda coordinated by the Institute of Tropical Medicine. This open-label study aimed to determine the safety of a contraceptive intravaginal ring (IVR) and to investigate the feasibility, acceptability and adherence to vaginal ring use in a group of Rwandan women. Chapter 4.2 was based on the cross-sectional baseline data of this study, whereas chapter 4.3, 4.4 and 4.5 were based on the longitudinal data of all visits of the Ring Plus study.

A total of 120 sexually active, HIV-negative women between 18 and 35 years old were randomised to an intermittent or a continuous regimen of ring use. The intermittent group used the contraceptive ring for three weeks, followed by one week without IVR before starting with a new ring. The continuous group used the IVR for three weeks, skipped the one-week-break, and immediately inserted a new ring for the next three weeks. The investigational product in this study was the NuvaRing® (Organon, Oss, the Netherlands). The ring contained 11.7 mg etonogestrel and 2.7 mg ethiynylestradiol which was released at an average amount of 0.102 mg and 0.0115 mg per 24 hours respectively, over a period of three weeks.

At the baseline visit, demographic information was collected, and vaginal, urinary and

Table 4.1: Baseline characteristics of Ring Plus participants, stratified by randomisation group

	Group A (intermittent use) n (%)	Group B (continuous use) n (%)	Total n (%)
Nugent score			
0-3	24 (40)	24 (41)	48 (40)
4-6	7 (12)	7 (12)	14 (12)
7-10	29 (48)	28 (47)	57 (48)
Chlamydia	4 (7)	6 (10)	10 (8)
Gonorrhea	2 (3)	5 (8)	7 (6)
HIV	0 (0)	0 (0)	0 (0)
HSV-2	21 (35)	26 (43)	47 (39)
Syphilis	3 (5)	3 (5)	6 (5)

blood sampling was done to determine baseline values. The following visit (for enrolment and randomisation) was planned on the first day of the participant's menstrual cycle. Follow-up visits coincided with the IVR removal and insertion times. At each ring removal visit, three vaginal swabs were collected and the used IVR was divided into three equal parts and stored according to the study procedures. One cotton swab was used to prepare slides for microscopy (wet mount, Nugent scoring and FISH) and two flocked swabs (Copan, Italy) were pooled together to characterise the vaginal microbiome with qPCR (Table 4.2). All participants were followed up for a maximum of 14 weeks and seen for five or six visits after the enrolment visit. [412]

At baseline, the median age of the study population was 28 years, 66% of the women had ever used hormonal contraception and 88% of the women had already delivered vaginally. Furthermore, an equal numbers of STIs was diagnosed in both randomisation groups. BV was also similarly distributed in both groups: 48% of the participants using the IVR intermittently and 47% of the participants following the continuous regimen were diagnosed with BV, according to the Nugent score, at the first study visit (Table 4.1).

Table 4.2: Ring Plus study procedures for both randomisation groups.
CM/AE: Concomitant Medication/Adverse Events; IDI: In-Depth Interview; IAQ: Interviewer Administered Questionnaire; FGD: Focus Group Discussion; VIA: Visual Inspection with Acetic acid

	Intermittent users			Continuous users		
		Ring in/out	Procedures		Ring in/out	Procedures
Prescreening			HIV/STI counselling Informed consent Collect contact- and menses-information			HIV/STI counselling Informed consent Collect contact- and menses-information
IDI			IDI			IDI
Baseline (Week 0)			Current medication Medical history Counselling Vaginal/physical exam Blood/ vaginal (swabs+CVL)/ urine samples IAQ			Current medication Medical history Counselling Vaginal/physical exam Blood/ vaginal (swabs+CVL)/ urine samples IAQ
Enrolment (Week 1)	V1	Ring 1 in	Counselling Randomisation Physical exam Urine sample	V1 (+-3 d)	Ring 1 in	Counselling Randomisation Physical exam Urine sample
Week 4	V2 (+2 d)	Ring 1 out	CM/AE/Counselling Vaginal/physical exam Vaginal (swabs)/ urine samples IAQ	V2 (+- 3d)	Ring 1 out 2 in	CM/AE/Counselling Vaginal/physical exam Vaginal (swabs)/ urine samples IAQ
Week 5	V3 (-2 d)	Ring 2 in	CM/AE/counselling			
Week 7				V3 (+-3d)	Ring 2 out 3 in	CM/AE/Counselling Vaginal/physical exam Vaginal (swabs)/ urine samples IAQ FGD
Week 8	V4 (+2 d)	Ring 2 out	CM/AE/Counselling Vaginal/physical exam Vaginal (swabs)/ urine samples IAQ FGD			
Week 9	V5 (-2 d)	Ring 3 in	CM/AE/counselling			
Week 10				V 4 (+-3 d)	Ring 3 out 4 in	CM/AE/Counselling Vaginal/physical exam Vaginal (swabs)/ urine samples IAQ
Week 12	V6 (+2 d)	Ring 3 out	CM/AE/Counselling Vaginal/physical exam VIA Vaginal (swabs+CVL)/ urine samples ACASI IAQ			
Week 13				V5 (+-3 d)	Ring 4 out	CM/AE/Counselling Vaginal/physical exam VIA Vaginal (swabs+CVL)/ urine samples ACASI IAQ
Week 14	V7 (+-3 d)		FGD IDI	V6 (+-3 d)		FGD IDI

4.2 Unravelling the bacterial vaginosis-associated biofilm: technical preparation

Abstract

Bacterial vaginosis, a condition defined by increased vaginal discharge without significant inflammation, is characterised by a change in the bacterial composition of the vagina. *Lactobacillus* spp., associated with a healthy vaginal microbiome, are outnumbered by BV-associated organisms. These bacteria could form a polymicrobial biofilm which allows them to persist in spite of antibiotic treatment. In this study, we examined the presence of *Gardnerella vaginalis* and *Atopobium vaginae* in vaginal biofilms using Peptide Nucleic Acid probes targeting these bacteria. For this purpose, we developed three new PNA probes for *A. vaginae*. The most specific *A. vaginae probe*, AtoITM1, was selected and then used in an assay with two existing probes, Gard162 and BacUni-1, to evaluate multiplex FISH on clinical samples. Using quantitative polymerase chain reaction as the gold standard, we demonstrated a sensitivity of 66.7% (95% confidence interval: 54.5% - 77.1%) and a specificity of 89.4% (95% confidence interval: 76.1% - 96%) of the new AtoITM1 probe. FISH enabled us to show the presence of a polymicrobial biofilm in bacterial vaginosis, in which *A. vaginae* is part of a *G. vaginalis*-dominated biofilm. We showed that the presence of this biofilm is associated with high bacterial loads of *A. vaginae* and *G. vaginalis*.

Adapted from:

Hardy L, Jespers V, Dahchour N, Mwambarangwe L, Musengamana V, Vaneechoutte M and Crucitti T. Unravelling the Bacterial Vaginosis-Associated Biofilm: a Multiplex *Gardnerella vaginalis* and *Atopobium vaginae* Fluorescence In Situ Hybridization Assay Using Peptide Nucleic Acid Probes. PLoS ONE 10(8): e0136658 (authentic paper in appendix).

4.2.1 Introduction

Bacterial vaginosis (BV), a condition characterised by increased vaginal discharge without significant inflammation, is highly prevalent in women of reproductive age. It increases the risk for acquisition and transmission of sexually transmitted infections, including HIV, and is associated with preterm birth in pregnant women [74, 75]. BV is a dysbiotic condition of unknown aetiology and is characterised by a change in the microbial composition of the vagina. *Lactobacillus* spp., associated with a healthy vaginal microbiome, are outnumbered by an array of BV-associated organisms including *Gardnerella vaginalis* [15,74,75]. However, several studies suggest that the mere presence of *G. vaginalis* is not sufficient for the diagnosis of BV. Indeed, *G. vaginalis* is also present in 50% to 70% of women without BV according to Nugent score [40,41,158]. *G. vaginalis* expresses various virulence factors such as vaginolysin [263] and sialidase [160]. It can also produce a biofilm [9], thereby increasing its tolerance to lactic acid and hydrogen peroxide produced by lactobacilli [413, 414] and to antimicrobial treatment [266, 267]. Furthermore, it has been suggested that its adherence and biofilm-forming capacities allow *G. vaginalis* to initiate the colonisation and scaffolding of the vaginal epithelium to which other species can attach subsequently [260, 264].

As was first shown a decade ago, *Atopobium vaginae* is one of the many other species that are characteristic of BV [163, 164, 167, 171, 330]. In one study, *A. vaginae* was detected in 80% of samples testing positive for *G. vaginalis* and made up 40% of the total biofilm mass dominated by *G. vaginalis* [9]. This association was confirmed in a study by Bradshaw et al. [170]: 93% of samples containing *A. vaginae* also contained *G. vaginalis*, whereas only 10% tested positive for *G. vaginalis* when *A. vaginae* was absent [322]. In contrast to *G. vaginalis*, *A. vaginae* is rarely part of the healthy vaginal microbiome and is considered a more specific marker of BV than *G. vaginalis* [163, 165, 170].

It is postulated that a biofilm provides bacteria with a competitive advantage over planktonic bacteria and that polymicrobial biofilms may offer additional advantages over single-species biofilms. Mechanisms that have been described in previous studies include metabolic cooperation, increased resistance to antibiotics or host immune responses [7] and an enlarged gene pool with more efficient sharing of genetic material compared to mono-species biofilms [348]. Polymicrobial coexistence is the dominant form in environmental biofilms, but is also prominent in the human body [7]. A well-known example is dental biomass: anaerobic bacteria, which are sensitive to oxygen, can survive and persist under the aerobic conditions in the oral cavity due to the consummation of oxygen by aerobic bacteria in the dental biofilm [374].

On the basis of these previous findings, we hypothesise that a polymicrobial biofilm consisting of *A. vaginae* and *G. vaginalis* and other bacteria not discussed in this study may serve as a marker of BV. Thus, better visualisation of the structure of vaginal biofilms

and identification of the bacterial components of the biofilm may contribute to better understanding of BV. To study the role of *A. vaginae* and *G. vaginalis* in BV, we designed and evaluated the performance of FISH with PNA probes for *A. vaginae* and *G. vaginalis*. Three new PNA probes were designed for *A. vaginae*. The most specific *A. vaginae* probe was selected, and subsequently used together with a PNA probe that had been described for the detection of *G. vaginalis* and a positive control probe that detects a broad range of bacteria in order to evaluate the multiplex FISH on clinical samples.

4.2.2 Methods and materials

Design of PNA probes

PNA probes targeting the bacterial 16S rRNA were synthesised by Panagene (Daejeon, South Korea). A fluorescent label was attached using a double 8-amino-3,6-dioxaoctanoic acid (AEEA) linker at the N terminus. We evaluated the performance of three new PNA probes for *A. vaginae*: Ato291, previously described as a DNA FISH probe [9, 415] and AtoITM1 and AtoITM2, which we developed using the Applied Biosystems PNA designer software (http://www6.appliedbiosystems.com/support/pnadesigner.cfm), based on sequences of species-specific PCR primers from Burton [166] for AtoITM1 and from Fredricks [195] for AtoITM2. The probes met the following criteria: purine content was limited to less than 60%; a maximum of four purines in a purine-stretch and a maximum of three guanines in a guanine-stretch were allowed; and self-complementarity was absent, considering that PNA/PNA interactions are stronger than PNA/DNA interactions. For the detection of *G. vaginalis*, a previously described probe, Gard162 [204, 205], was used. The broad-range bacterial probe, BacUni-1, previously designed [416] as a modified version of the broad-range eubacterial DNA probe [417], was used as a positive control. The probe specifications are listed in Table 1.

Table 4.3: Probe specifications

Name	Target	Probe sequence (5'-3')	Tm (°C)	%GC	Reference
AtoITM1	*Atopobium vaginae*	Alexa488-OO-CTC-CTG-ACC-TAA-CAG-ACC	66	55.6	Newly designed, based on Burton et al. [166]
AtoITM2	*A. vaginae*	Alexa488-OO-GCG-GTY-TGT-TAG-GTC-AGG	72	58.3	Newly designed, based on Fredricks et al. [195]
Ato291	*A. vaginae*	Alexa488-OO-GGT-CGG-TCT-CTC-AAC-CC	68	60.0	Newly designed, based on Harmsen et al. [415]
Gard162	*Gardnerella vaginalis*	Alexa647-OO-CAG-CAT-TAC-CAC-CCG	61	60.0	Machado et al. [204]
BacUni-1	Eubacteria	Alexa555-CTG-CCT-CCC-GTA-GGA	64	66.7	Perry-O'Keefe et al. [416]

Bacterial culture techniques for evaluation performance PNA probes

The performance of the PNA probes was evaluated using clinical isolates, obtained from the collections of the Institute of Tropical Medicine (ITM) and Ghent University. The five most frequently detected *Lactobacillus* species [3,15], representing the non-BV microbiome, were chosen as a negative control to assure that the probes would not cross-hybridise with the normal microbiome (Table 4.4). Furthermore we selected the most frequent BV-associated bacteria (8 *A. vaginae* strains, 5 *G. vaginalis* strains) as a negative control for *G. vaginalis* and *A. vaginae* respectively in addition to 2 *Prevotella melaninogenica* strains (Table 4.4).

Table 4.4: Specificity testing in duplicate of peptide nucleic acid (PNA) probes using cultured bacteria

Species	Strain	AtoITM1	AtoITM2	Ato291	Gard162	BacUni-1
Atopobium vaginae	CCUG 38953^T	+[1]	+	+	-[2]	+
Atopobium vaginae	UG080499	+	+	+	-	+
Atopobium vaginae	UG071164	+	+	+	-	+
Atopobium vaginae	UG020349	+	+	+	-	+
Atopobium vaginae	UG160373	+	+	+	-	+
Atopobium vaginae	UG550940	+	+	+	-	+
Atopobium vaginae	UG030313	+	+	+	-	+
Atopobium vaginae	UG030312	+	+	+	-	+
Gardnerella vaginalis	UG860108	-	-	-	+	+
Gardnerella vaginalis	UG030406	-	-	-	+	+
Gardnerella vaginalis	UG860107	-	+	+	+	+
Gardnerella vaginalis	LMG 7832^T	-	+	+	+	+
Gardnerella vaginalis	UG030407	-	+	+	+	+
Lactobacillus iners	LMG 18914^T	-	-	+	-	+
Lactobacillus vaginalis	LMG 12891^T	-	-	+	-	+
Lactobacillus jensenii	LMG 6414^T	-	-	+	-	+
Lactobacillus crispatus	LMG 9479^T	-	-	+	-	+
Lactobacillus gasseri	LMG 9203^T	-	-	+	-	+
Prevotella melaninogenica	UG160361	-	-	+	-	+
Prevotella melaninogenica	UG040818	-	-	-	-	+

The signal was considered positive if it had a positive counterpart in the DAPI stain and displayed a positive signal simultaneously with the broad-range probe. The signal was considered negative if no signal was seen with the species-specific probe.

[1](+) Presence of hybridisation

[2](−) Absence of hybridisation

This small test panel is a limitation of the study and more expansive testing will be required to assure that there is no cross-reactivity with other bacteria. The strains from frozen stocks in skimmed milk (-80 °C) were cultured on Columbia agar base (Becton Dickinson Biosciences, Erembodegem, Belgium) + 5% horse blood and grown under anaerobic conditions (10% hydrogen, 10% carbon dioxide and 80% nitrogen), using an anaerobic incubator (Whitley DG250) at 37 °C for 48 h and bacteria were streaked onto fresh plates every 48-72 hours. To determine the limit of detection, FISH was performed

using the AtoITM1, Gard162 and BacUni-1 probe on serial tenfold dilutions using the fixative used throughout the study: Carnoy solution (6:3:1, ethanol:chloroform:glacial acetic acid [418, 419]). Carnoy solution was chosen as a fixative solution because of its proven effectiveness in the stabilisation of and minimal shrinkage in tissue structure [419, 420]. The concentration of the dilutions was estimated based on the turbidity of the sample compared to McFarland Standards (Bio-Merieux SA, Marcy l'Etoile, France) and ranged from $1.2x10^9$ to $1.2x10^3$ cells per ml. The suspensions were vortexed briefly and 5 µl was spotted into the hybridisation chamber; a 5 mm^2 area marked with a PAP Pen (Sigma Aldrich, St. Louis, USA) that creates a hydrophobic border on a Superfrost Plus slide (Menzel-Gläser, Braunschweig, Germany). The spotted suspensions were dried before performing FISH. Experiments were performed in duplicate.

Clinical samples

Ethics statement Vaginal samples were collected from 119 women participating in a clinical trial in Rwanda studying the vaginal microbiome and acceptability of a contraceptive ring (the 'Ring Plus' study, ClinicalTrials.gov identifier NCT01796613) (data analysis on-going) [412]. Participants were between 18 and 35 years old and provided written informed consent for participation in the study. The Ring Plus study and consent procedure were approved by the Rwanda National Ethics Committee, Rwanda; the Institutional Review Board of the ITM Belgium; and the ethics committee of the University Teaching Hospital in Antwerp, Belgium.

Vaginal sample collection and preparation Vaginal sampling was carried out by the study clinician as part of the study procedures. Two Copan flocked swabs (Copan, Brescia, Italy) and one cotton swab were brushed against the lateral walls of the vagina. The cotton swab was immediately rolled on a Superfrost Plus slide (Menzel-Gläser) which was heat-fixed by passing twice through a flame. The Superfrost Plus (Menzel-Gläser) slides were stored for maximum six months and shipped to ITM at room temperature and fixed for a minimum of 12 hours at ITM, submerged in Carnoy solution [418, 419]. The Copan flocked swabs were eluted by vortexing each swab for at least 15 seconds in 1.2 ml of diluted phosphate buffered saline (PBS) (pH 7.4 - 1:9, PBS:saline). The two eluates were combined and divided into three aliquots, which were stored at -80 °C. The swab eluates were shipped frozen (-191 °C) in a dry shipper to the ITM to determine the total bacterial load of *A. vaginae* and *G. vaginalis* by means of qPCR.

Urine sample collection and preparation According to an earlier described procedure [305], first-void urine was collected by the participants and 2 ml was transferred

immediately to a 15 ml tube containing 2 ml of Carnoy solution. The sample was fixed overnight and after centrifugation (10 minutes at 3200 g), the supernatant was decanted and the pellet was treated for a second time with 0.75 µl of Carnoy solution. The samples were stored between 2-8 °C and shipped at room temperature to the ITM. Before applying FISH, the urine samples were vortexed briefly and 5 µl was spotted into the hybridisation chamber on a Superfrost Plus slide (Menzel-Gläser).

Quantitative polymerase chain reaction for quantification of bacteria in vaginal samples

DNA was extracted from 250 µl of the vaginal swab eluate using the Abbott m2000sp automated extraction platform (Abbott, Maidenhead, UK), according to the manufacturer's instructions. The volume of 200 µl DNA extract was stored at -80 °C until testing. qPCR was performed for each bacteria species separately, to avoid competition between the primers. The 25 µl PCR mixture contained 12.5 µl Rotor-Gene SYBR Green RT-PCR Master mix (Qiagen, Venlo, the Netherlands), 5 µl DNA extract, 0.5 µM of *A. vaginae* or 1 µM of *G. vaginalis* forward and reverse primers (Integrated DNA Technologies, Leuven, Belgium) and RNase-free water provided with the Rotor-Gene SYBR Green PCR kit. The primers for *A. vaginae* and *G. vaginalis* and the amplification reactions (Rotor Gene Q MDx 5 plex, Qiagen) have been described before [3].

Quantification was done using standard curves, constructed using DNA extracts from *A. vaginae* (CCUG 38953T) and *G. vaginalis* (LMG 7832T), grown at 35 °C ± 2 °C on Columbia agar base (Becton Dickinson) + 5% horse blood, under anaerobic conditions. DNA concentrations were determined using NanoDrop (Thermo Fisher Scientific, Erembodegem, Belgium) and the number of genomes was calculated using the described genome sizes and G+C content of the strains. A total of six tenfold dilutions of the DNA stocks were prepared in high performance liquid chromatography (HPLC) grade water. Both the standard curve and samples were run in duplicate. The bacterial load was expressed as genome equivalents (geq)/ml.

Peptide nucleic acid fluorescence in situ hybridisation procedure

Multiplex hybridisation was performed on a Superfrost Plus slide (Menzel-Gläser) in a 5 mm^2 quadrant hybridisation area marked with a PAP pen (Sigma Aldrich, St. Louis, USA), a liquid-repellent slide marker. The slide was covered with a cover slip after addition of a hybridisation buffer that contained 200 nM of each probe: species-specific probes for *A. vaginae* (AtoITM1 or AtoITM2 or Ato291) and *G. vaginalis* (Gard162), and the broad-range BacUni-1 probe. The hybridisation solution consisted of 10% (wt/vol) dextran

sulphate (Sigma Aldrich), 10 mM NaCl (Merck KGaA, Darmstadt, Germany), 2% (vol/vol) formamide (Merck KGaA), 0.1% (wt/vol) sodium pyrophosphate (Sigma Aldrich), 0.2% (wt/vol) polyvinylpyrrolidone (Sigma Aldrich), 0.2% (wt/vol) Ficoll (Sigma Aldrich), 5 mM disodium EDTA (Merck KGaA), 0.1% (vol/vol) Triton X-100 (Acros Organics, Geel, Belgium) and 50 mM Tris-HCl at pH 7.5 (Sigma Aldrich).

The slides were incubated in a hybridisation oven (Shake 'N Bake, Boekel Scientific, Feasterville, Pennsylvania) in humid conditions, which were achieved by adding a small tray of water, at 60 °C for 60 minutes. After the slides were rinsed with double-distilled (dd) H_20, they were immersed in a washing solution containing 5 mM Tris base, 15 mM NaCl and 0.1% (vol/vol) Triton X-100 (at pH 10) for 15 min at 60 °C on the rocking shelves of the hybridisation oven. After this washing step, the slides were rinsed again with ddH20 and air-dried in the dark at room temperature. Subsequently, the slides were counterstained with 6-diamidine-2-phenylindole dihydrochloride (DAPI) (Serva, Heidelberg, Germany), a DNA-intercalating agent that stains the chromosomes of both prokaryotic and eukaryotic cells, for 5 minutes at room temperature in the dark and rinsed with ddH_20. Before imaging, the slides were air-dried at room temperature in the dark.

Assessment of reproducibility of fluorescence in situ hybridisation

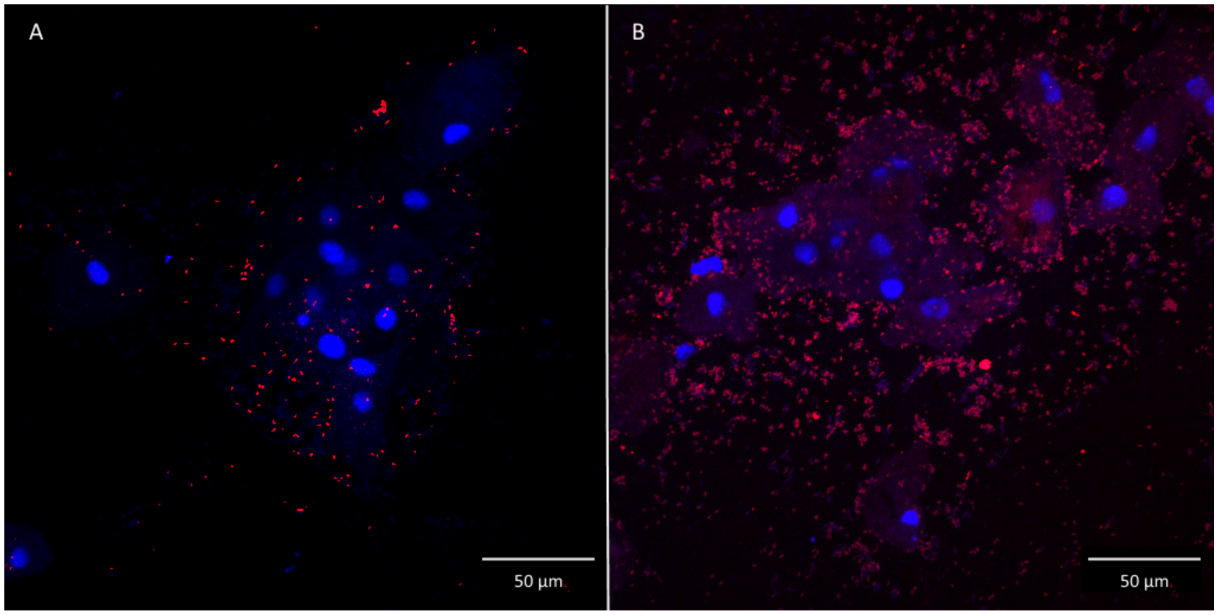
The inter-run repeatability of FISH was evaluated by comparing the outcomes of two independent FISH runs for a subset of the samples. The second hybridisation was performed 6 months later on a subset of 15% of the samples (N=17), which reflects one FISH run. We selected 17 samples showing variable results for the species-specific signal and positive for the broad-range probe. A new hybridisation spot was drawn and fresh hybridisation and washing buffer was used on the exact same slides used in the first FISH run. Visual inspection by confocal microscopy was performed by the same microscopist.

Microscopic analysis of hybridised samples

The hybridised samples were stored in the dark at room temperature for a maximum of one week before microscopic observation using confocal laser scanning microscopy (CLSM) (LSM700, Zeiss, Oberkochen, Germany). The microscope operates with four stable, solid-state lasers at wavelengths of 405 to 639 nm, and is therefore able to detect all three fluorescently labelled probes and the DAPI stain at once in one hybridisation chamber. The sample was first scanned at 100X magnification (objective: EC Plan-Neofluar 10x/0.30 Ph1 M27), before individual bacteria were identified at 400X magnification (objective: Plan-Apochromat 40x/1.3 Oil Ph3 M27). Separate scattered bacterial cells were defined as dispersed bacteria (Fig 4.1A). Aggregates of bacterial cells attached to the vaginal epithelial

cells were defined as adherent bacteria forming a biofilm (Fig 4.1B). The species-specific signal was considered positive only if it had a positive counterpart in the DAPI stain and if it displayed a positive signal simultaneously with the broad-range probe.

Figure 4.1: Dispersed bacteria versus biofilm. Confocal laser scanning images with 400x magnification of *Gardnerella vaginalis* biofilm in 2 vaginal slides (A and B) in a superimposed image: vaginal epithelial cells DAPI in blue and *G. vaginalis* specific PNA-probe Gard162 with Alexa Fluor 647 in red. A: vaginal sample with dispersed bacteria; B: vaginal sample with bacteria in biofilm.



Statistical analysis

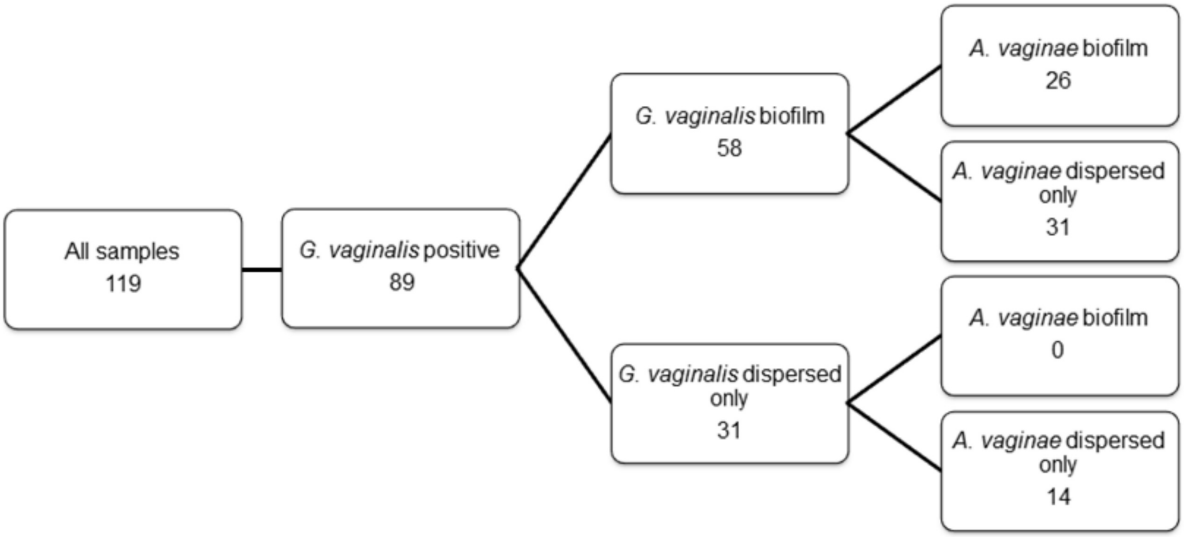
The specificity and sensitivity of FISH for vaginal slides was compared with the qPCR as a reference method. Bacterial counts were log 10 transformed before analysis. Data analysis was done using STATA13. The p-values reported for associations between the presence of bacterial species/biofilm and bacterial loads from the qPCR results originate from the non-parametric Kruskal-Wallis equality-of-populations rank test.

4.2.3 Results

Peptide nucleic acid fluorescence in situ hybridisation probe performance on bacterial strains

The three probes specific for *A. vaginae* clearly hybridised with all eight *A. vaginae* strains tested (Table 4.4). However, Ato291 showed cross-hybridisation with three *G. vaginalis* strains, with all *Lactobacillus* species and with one *Prevotella* strain. The newly developed AtoITM2 probe cross-hybridised with three *G. vaginalis* strains. Only AtoITM1 performed

Figure 4.2: Distribution of samples according to fluorescence in situ hybridisation (FISH). Aggregates of bacterial cells attached to the vaginal epithelial cells, were defined as biofilm. Separate scattered bacterial cells, without the presence of biofilm, were defined as dispersed only bacteria.



without false positive results and was selected for further evaluation on the clinical samples. The Gard162 probe was able to identify all five *G. vaginalis* test strains and showed no cross-hybridisation with any of the other 15 species tested (Table 4.4). All bacterial strains tested hybridised with the broad-range BacUni-1 probe. According to the FISH results of the serial dilutions, the limit of detection for AtoITM1, Gard162 and BacUni-1 probes was 1.2 x 10^5 cells per ml.

Detection of *Atopobium vaginae* and *Gardnerella vaginalis* in clinical samples by Peptide nucleic acid fluorescence in situ hybridisation

In a small pilot study, a total of 10 paired vaginal slides and urine samples were tested to evaluate the suitability of each type of sample for FISH analysis. Six out of 10 urine samples could not be assessed due to the low presence of vaginal epithelial cells in the urine, whereas this problem was experienced in only 2 vaginal samples. Therefore, it was decided to continue analyses on vaginal slides only.

Using the AtoITM1 PNA-probe, *A. vaginae* was visualised as dispersed entities, without the presence of biofilm, in 27/119 (22.7%) of the samples. *A. vaginae* biofilm was present in 26/119 (21.8%) samples. *A. vaginae* FISH was negative in the remaining 66/119 (55.5%) samples. PNA-FISH using Gard162 detected dispersed-only *G. vaginalis* in 31/119 (26%) samples, *G. vaginalis* biofilm in 58/119 (48.7%) samples (e.g., Fig 4.2) and 30/119 (25.3%) samples were negative for *G. vaginalis*. Of the 89 *G. vaginalis* FISH-positive samples (dispersed or biofilm), 36 samples (41%) were negative for *A. vaginae*. However, all samples with *A. vaginae* biofilm showed a *G. vaginalis* biofilm as well (e.g., Fig 4.3, 4.4, 4.5).

Figure 4.3: *Gardnerella vaginalis* biofilm. Montage of confocal laser scanning images with 400x magnification of *G. vaginalis* biofilm, negative for *Atopobium vaginae*, in 4 vaginal samples (A-D) in a superimposed image: vaginal epithelial cells DAPI in blue and *G. vaginalis* specific peptide nucleic acid (PNA)-probe Gard162 with Alexa Fluor 647 in red. For clarity we omitted the BacUni-1 plane; the bacteria that did not hybridise with Gard162 are visible in DAPI blue.

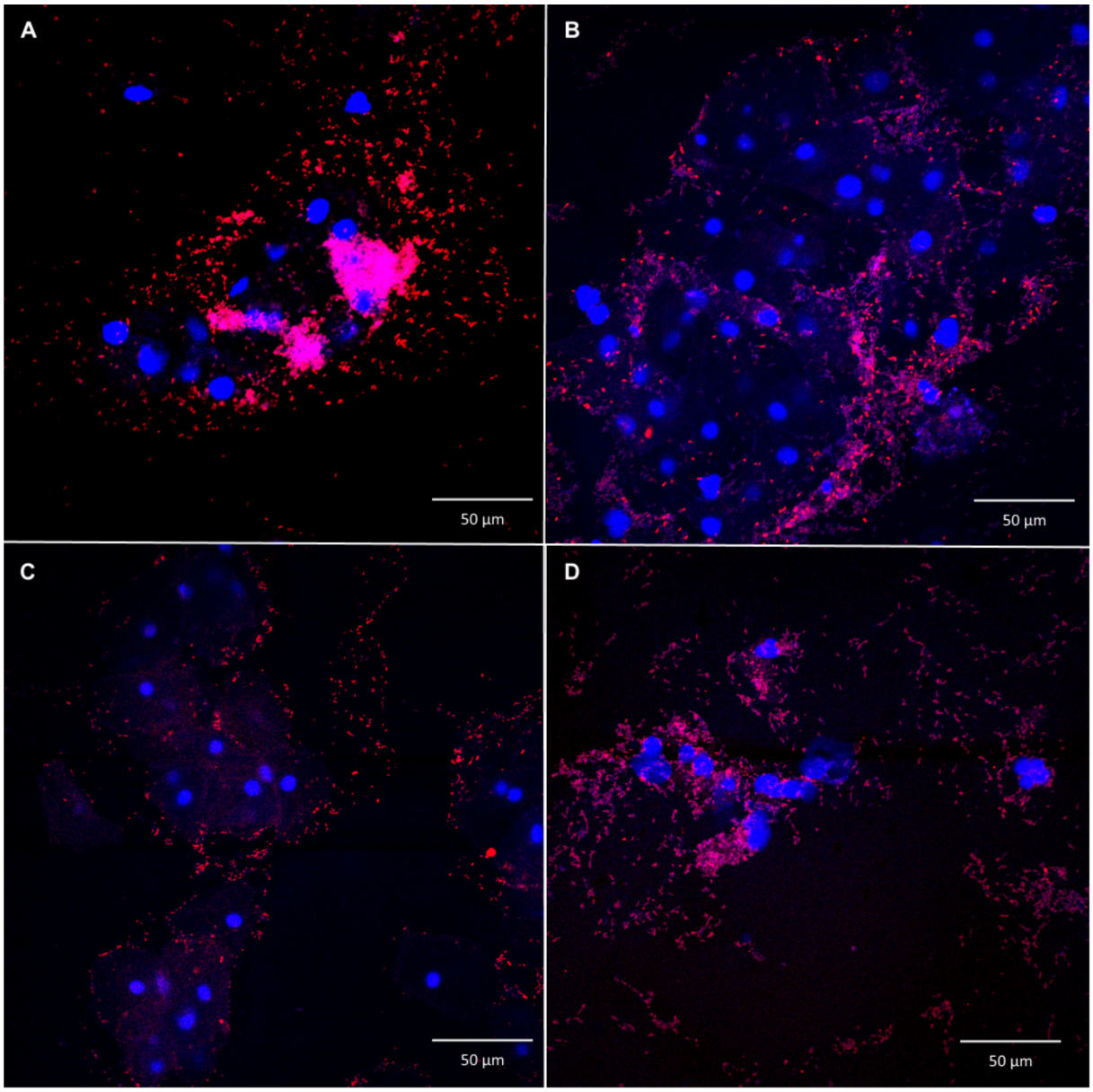


Figure 4.4: Superimposed image of polymicrobial biofilm of *Atopobium vaginae* and *Gardnerella vaginalis*. Montage of confocal laser scanning microscopy images with 400x magnification of polymicrobial biofilm in 6 vaginal samples (A-F) in a superimposed image: vaginal epithelial cells DAPI in blue, *G. vaginalis* specific PNA-probe Gard162 with Alexa Fluor 647 in red and *A. vaginae* specific PNA-probe AtoITM1 with Alexa Fluor 488 in green. For clarity we omitted the BacUni-1 plane; the bacteria that are not bound by the specific probes are visible in DAPI blue.

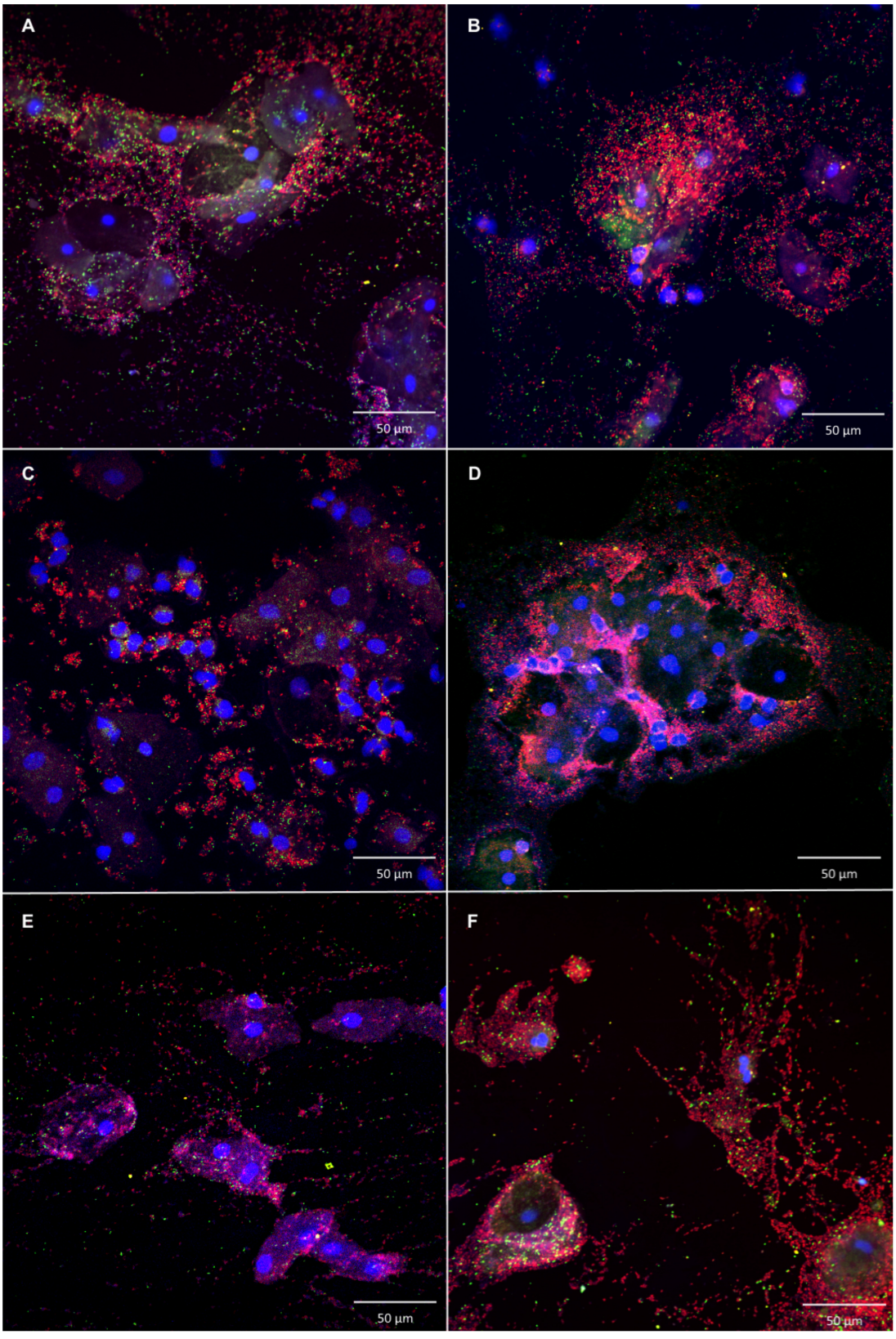
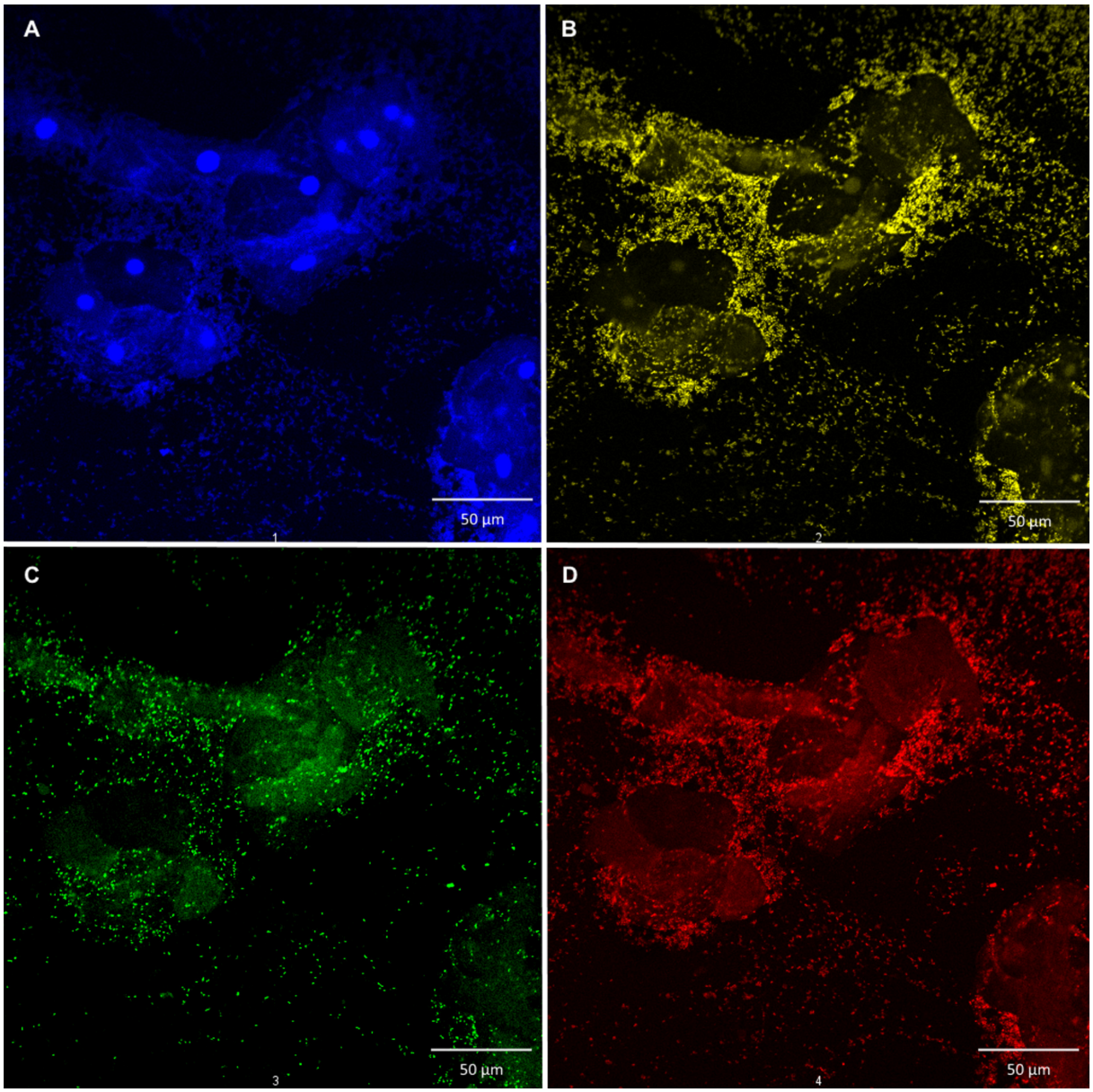


Figure 4.5: Polymicrobial biofilm of *Atopobium vaginae* and *Gardnerella vaginalis* in different panes. Confocal laser scanning image with 400 x magnification of polymicrobial biofilm in different panes, A: vaginal epithelial cells DAPI in blue, B: all bacteria, BacUni-1 PNA-probe with Alexa Fluor 555 in yellow, C: *A. vaginae* specific PNA-probe AtoITM1 with Alexa Fluor 488 in green, D: *G. vaginalis* specific peptide nucleic acid (PNA)-probe Gard162 with Alexa Fluor 647 in red (superimposed image can be seen in Fig. 3A).



Characterisation of vaginal samples by quantitative polymerase chain reaction

A total of 119 vaginal samples were available for qPCR analysis. *A. vaginae* was present in 72 (60%) of the samples with a mean log of 7.55 ± 1.34 geq/ml. *G. vaginalis* was detected in 95 (80%) of the samples with a mean log of 7.38 ± 1.11 geq/ml.

Performance of probes in vaginal samples

Quantitative PCR was used as the reference method for detection and quantification of *A. vaginae* and *G. vaginalis*. The signal of the species-specific probes was only considered positive if a positive counterpart was seen in the DAPI stain and with the universal BacUni-1 probe. When assessing the results with FISH probe AtoITM1 against the qPCR outcomes for *A. vaginae* for 119 vaginal samples, FISH results were false negative for 24 samples and false positive for 5 samples, resulting in a sensitivity of 66.7% (95% confidence interval (CI): 54.5% - 77.1%) and a specificity of 89.4% (95% CI: 76.1% - 96%) (Table 4.5). The mean log for the true positive samples (positive with qPCR and FISH) was 7.73 geq/ml, as compared to a mean log of 7.19 geq/ml for the false negative FISH results (p=0.399). For Gard162, the *G. vaginalis* probe, 13 FISH results were false negative and six false positive. The sensitivity was 86.3% (95% CI: 77.4% - 92.2%) and the specificity 75.0% (95% CI: 52.9% - 89.4%) (Table 4.5). The mean log for the true positive results for *G. vaginalis* was 7.61 geq/ml compared to a mean log of 5.94 geq/ml for the false negative results (p<0.001).

Table 4.5: Performance of *Atopobium vaginae* (AtoITM1) and *Gardnerella vaginalis* (Gard162) peptide nucleic acid probes, compared to quantitative polymerase chain reaction (qPCR) results, for 119 vaginal slides

FISH	**qPCR**				
	A. vaginae positive	*A. vaginae* negative	*G. vaginalis* positive	*G. vaginalis* negative	Total
AtoITM1 positive	48 (66.7%)	5 (10.6%)			53
AtoITM1 negative	24 (33.3%)	42 (89.4%)			66
Gard162 positive			82 (86.3%)	6 (25.0%)	88
Gard 162 negative			13 (13.7%)	18 (75.0%)	31
Total	72	47	95	24	

Assessment of the repeatability was done using 17 samples. After the first hybridisation, all samples showed a signal for the BacUni-1 probe, 5 and 9 samples out of 17 for the AtoITM1 and Gard162 probe respectively. The results of the second FISH with the BacUni-1 and Gard162 probe were in full agreement with the first run. For the AtoITM1 probe, only one sample had a different result in the second run (negative at first, but positive in the second run).

The presence of biofilm related to bacterial loads

The probability of detecting bacteria in a biofilm with FISH was higher when high ($>10^6$ geq/ml) bacterial loads for *G. vaginalis* ($p<0.001$) and *A. vaginae* ($p<0.001$) were present. The mean log of both species was highest when *A. vaginae* was part of the biofilm, compared to a biofilm of *G. vaginalis* only. *A. vaginae* biofilm was always observed together with *G. vaginalis* (Table 4.6).

Table 4.6: Presence of *Atopobium vaginae* and *Gardnerella vaginalis*, as assessed by fluorescence in situ hybridisation (FISH), in relation to *A. vaginae* and *G. vaginalis* loads as determined by quantitative polymerase chain reaction for 119 vaginal samples

	Total	*G. vaginalis* **count 0**	*G. vaginalis* **count<10^6 geq/ml**	*G. vaginalis* **count>10^6 geq/ml**	*G. vaginalis* **mean log**	*A. vaginae* **count 0**	*A. vaginae* **count<10^6 geq/ml**	*A. vaginae* **count>10^6 geq/ml**	*A. vaginae* **mean log**
Detected by PNA FISH		N (%)	N (%)	N (%)	geq/ml	N (%)	N (%)	N (%)	geq/ml
A. vaginae									
Absent	66	20 (30.3)	13 (19.7)	33 (50.0)	4.87	42 (63.6)	7 (10.6)	17 (25.8)	2.55
Dispersed only	27	3 (11.1)	0 (0.0)	24 (88.9)	6.82	3 (11.1)	4 (14.8)	20 (74.1)	6.21
Biofilm[1]	26	1 (3.9)	1 (11.8)	24 (92.3)	7.50	2 (7.7)	0 (0)	24 (92.3)	7.66
G. vaginalis									
Absent	30	17 (56.7)	6 (20.0)	7 (23.3)	2.57	24 (80.0)	3 (10.0)	3 (10.1)	1.05
Dispersed only	31	2 (6.5)	6 (19.4)	23 (74.2)	6.68	13 (41.9)	5 (16.1)	13 (41.9)	3.97
Biofilm	58	5 (8.6)	2 (3.5)	51 (87.9)	7.18	10 (17.2)	3 (5.2)	45 (77.6)	6.55

[1] *A. vaginae* biofilm = polymicrobial biofilm consisting of *A. vaginae* and *G. vaginalis*; No slides had *A. vaginae* biofilm only.

4.2.4 Discussion

We set out to evaluate the performance of PNA FISH for the investigation of the vaginal polymicrobial biofilm consisting of *G. vaginalis* and *A. vaginae*. For this purpose, we evaluated three newly designed *A. vaginae* PNA probes for their specificity and applied the most specific one, AtoITM1, on a range of fixed vaginal slides together with an already existing *G. vaginalis* and broad-range PNA probe.

Hybridisation-based techniques such as FISH have been used in various disciplines, such as cytogenetics and microbiology, to detect the presence or absence of nucleic acid sequences. Detection of DNA and RNA is generally done using DNA probes but the use of PNA probes is increasing. PNA molecules have a neutral backbone giving them a significant advantage in low ionic-strength conditions compared to DNA probes [208]. Low ionic-strength conditions prevent the complementary genomic sequences from reannealing when performing the FISH procedures; they facilitate denaturation of RNA secondary structures and favour hybridisation of the PNA probes with nucleic acids. In combination with the superior penetration of PNA probes through the cell wall and hydrophobic bilayer of the target organism [207], PNA FISH is a fast, simple and robust assay. We compared DNA

and PNA probes (data not reported) and can confirm that PNA FISH is faster and more robust than DNA FISH. In the current study, PNA FISH proved to be highly efficient for the identification and visualisation of the spatial arrangement of *A. vaginae* and *G. vaginalis* in the BV-associated biofilms. Moreover, PNA FISH showed excellent inter-assay repeatability for all three probes used.

Peptide nucleic acid fluorescence in situ hybridisation probe performance on bacterial strains

For the design of the PNA probes, we opted for Alexa fluorochromes, which have similar spectral properties as other fluorochromes, such as cyanine dyes, but are brighter and more resistant to photo bleaching [421].

PNA probes that specifically target *A. vaginae* have not been described before. The only probe thus far reported was a DNA probe (Ato291) [415]. The probe was designed to detect bacteria belonging to the *Atopobium* cluster in faecal samples. However, in silico evaluation of the specificity of the Ato291 probe showed cross-hybridisation with other bacteria belonging to the Coriobacteriaceae, a family of Actinobacteria, to which *A. vaginae* belongs. This is not surprising, since the probe was originally designed on the basis of sequences of Coriobacteriaceae strains isolated from faeces and clinical material. The probe has been used for the detection of *A. vaginae* in vaginal samples by Swidsinski et al. [9], but their findings have not yet been confirmed by other groups. In our experiments, using a PNA equivalent of the Ato291 probe, we showed a low specificity of the Ato291 probe on vaginal clinical isolates. The Ato291 probe cross-hybridised with three out of five *G. vaginalis* strains and all five *Lactobacillus* species. Therefore, we designed two new probes for *A. vaginae* targeting the 16S rRNA-gene, based on published PCR primers [166, 195] and we adjusted the sequences to fit the requirements for PNA probes. One of the new probes, AtoITM1, did not cross-react to any of the tested species and was further used for detection of *A. vaginae* in vaginal slides.

Gard162 is the first PNA FISH probe designed specifically for *G. vaginalis* and has extensively been tested by Machado et al. on a variety of cultured bacterial strains and clinical samples [204, 205]. Using this probe, we obtained clear hybridisation for all *G. vaginalis* isolates tested and observed no cross-reaction with strains of the other species tested, confirming the findings of Machado et al. [204].

Peptide nucleic acid fluorescence in situ hybridisation probe performance on clinical samples

Vaginal slides proved to be a valid sample type for imaging of the composition of the vaginal microbiome, if processed directly after sampling, as shown by Peltroche-Llacsahuanga et al. [422]. Collection of a vaginal swab is an easy and cheap sampling method, with a minimal burden on the study participant or patient. After heat fixation, we recorded that the slides can be stored at room temperature for up to at least six months and can be easily transported. A high quality vaginal sample can be obtained by thinly rolling the swab onto the slide. A thick vaginal smear on the contrary where the material is smeared onto the slide is not useful for FISH and microscopic visualisation.

The probes were also applied to 119 vaginal slides from women for whom the bacterial loads of *G. vaginalis* and *A. vaginae* had been quantified by qPCR. qPCR was used in this study as the reference method to evaluate the performance of the FISH probes, although comparison of these methods is subject to some limitations. qPCR is highly sensitive and was performed on a homogenised DNA extract representing half of the full sample. FISH however was carried out on 0.5 mm^2 of a vaginal slide, which could be heterogeneous. This is inevitably an underrepresentation of the vaginal sample. Both techniques were also performed using two different vaginal swabs, but the first collected specimen was used to prepare the slide as per study protocol.

After hybridisation for 60 minutes and washing for 15 minutes, both at 60 °C, the AtoITM1 probe gave only five false positive results, compared to qPCR results, resulting in a specificity of 90% for this set of samples. However, 24 samples that were positive according to the qPCR were not detected by FISH, which gives a sensitivity of 67%. These results are comparable to the sensitivity observed for vaginal samples using FISH for detection of Group B *Streptococcus* [422]. The authors of this study obtained a higher sensitivity after extraction of the swabs by centrifugation of the swab head in a NaCl solution [422], but this method would probably disrupt the epithelial biofilm and thus prevent us from investigating the organisation of the bacterial biofilm.

The relatively low sensitivity of the *A. vaginae* FISH assay cannot be explained by the bacterial load as measured by qPCR; the mean log of the true positive and false negative samples was not significantly different (log 7.73 and 7.19 geq/ml respectively). One possible explanation could be the typical structure of a biofilm, whereby an oxygen gradient exists from the top to the centre of the biofilm [423]. Anaerobic bacteria such as *A. vaginae* are possibly found more embedded in the biofilm, to take advantage of the anaerobicity. It could be that the PNA probes are not able to fully penetrate into the inner parts of the biofilm; or that if the PNA probes do penetrate, the fluorescence could be masked and not detected due to low resolution of the equipment.

For *G. vaginalis*, 6 false positive results and 13 false negative results were found compared to 95 positive and 24 negative samples according to qPCR. This implies a sensitivity of 86% and specificity 75% for the detection of *G. vaginalis* by the Gard162 probe using our FISH protocol for this set of samples. This is lower than reported by Machado [204], who, using the same probe, showed a full agreement between qPCR and FISH results for 13 vaginal samples. We were not able to elucidate why these discordant results were obtained.

Bacterial loads and the presence of a biofilm

Our study shows that higher bacterial loads of *G. vaginalis* and *A. vaginae*, as detected by qPCR, are associated with a higher probability of presence of a bacterial biofilm. Both bacterial species are important constituents of the vaginal epithelial biofilm [9, 332]. No samples contained *A. vaginae* in the absence of *G. vaginalis*, but almost half of the *G. vaginalis*-positive samples did not contain *A. vaginae* according to FISH results. Both bacteria were seen in a dispersed and an adherent state, but *A. vaginae* was always accompanied by *G. vaginalis*. The mere presence of *A. vaginae* did not simply predispose to a polymicrobial biofilm, but when *A. vaginae* was part of the biofilm, compared to a biofilm of only *G. vaginalis*, both bacterial species were present in higher concentrations.

We hypothesise that *G. vaginalis* is one of the main initiators of a vaginal biofilm, when it is present in high amounts. This vaginal biofilm creates a favourable environment for anaerobic bacteria, such as *A. vaginae*. One reason for the appearance of *A. vaginae* may be the presence of an oxygen gradient within the biofilm. By embedding itself within the biofilm, *A. vaginae* can take advantage of the anaerobicity, proliferates and exists in a mutualistic relationship with *G. vaginalis*.

4.2.5 Conclusion

Our study confirms that PNA FISH is a valuable tool for detecting and visualising biofilm-associated organisms in vaginal slides. This study describes the design and evaluation of a new PNA probe, AtoITM1, which can be included in multiplex FISH in BV biofilm research. Using the new probe, we have demonstrated the presence of a polymicrobial biofilm, with *A. vaginae* taking part in a *G. vaginalis* dominated biofilm.

4.3 A fruitful alliance: the synergy between *Atopobium vaginae* and *Gardnerella vaginalis*

Abstract

Bacterial vaginosis is characterised by a change in the microbial composition of the vagina. The BV-associated organisms outnumber the health-associated Lactobacillus species and form a polymicrobial biofilm on the vaginal epithelium, possibly explaining the difficulties with antibiotic treatment. A better understanding of vaginal biofilm with emphasis on *Atopobium vaginae* and *Gardnerella vaginalis* may contribute to a better diagnosis and treatment of BV. To this purpose, we evaluated the association between the presence of both bacteria by fluorescence in situ hybridisation and BV by Nugent scoring in 463 vaginal slides of 120 participants participating in a clinical trial in Rwanda. A bacterial biofilm was detected in half of the samples using a universal bacterial probe. The biofilm contained *A. vaginae* in 54.1% and *G. vaginalis* in 82.0% of the samples. *A. vaginae* was accompanied by *G. vaginalis* in 99.5% of samples. The odds of having a Nugent score above 4 was increased for samples with dispersed *G. vaginalis* and/or *A. vaginae* present (odds ratio 4.5; CI: 2-10.3). The probability of having a high Nugent score was even higher when a combination of adherent *G. vaginalis* and dispersed *A. vaginae* was visualised (odds ratio 75.6; CI: 13.3-429.5) and highest when both bacteria were part of the biofilm (odds ratio 119; CI: 39.9-360.8). Our study, although not comprehensive at studying the polymicrobial biofilm in BV, provided a strong indication towards the importance of *A. vaginae* and the symbiosis of *A. vaginae* and *G. vaginalis* in this biofilm.

Adapted from:

Hardy L, Jespers V, Abdellati S, De Baetselier I, Mwambarangwe L, Musengamana V, van de Wijgert J, Vaneechoutte M and Crucitti T. A fruitful alliance: the synergy between *Atopobium vaginae* and *Gardnerella vaginalis* in bacterial vaginosis-associated biofilm. STI 2016;0:1–5,pii: sextrans-2015-052475,doi: 10.1136 (authentic paper in appendix).

4.3.1 Introduction

Bacterial vaginosis (BV) is the most prevalent vaginal disorder in women of reproductive age. It increases the risk of acquisition and transmission of sexually transmitted infections, including HIV, and is associated with preterm birth in pregnant women [89, 127, 424]. The condition is characterised by a change in the microbial composition of the vagina: the *Lactobacillus* spp., associated with a healthy vaginal microbiome, are outnumbered by microaerophilic and anaerobic organisms, including *Gardnerella vaginalis* [15, 41, 127, 174, 260]. The mere presence of *G. vaginalis*, however, is not sufficient for the diagnosis of BV using traditional diagnostic algorithms (see below) because many women without BV also have *G. vaginalis* in their vaginal microbiome [15]. BV is, however, associated with high counts of *G. vaginalis* using molecular methods and/or the presence of a *G. vaginalis*-containing polymicrobial biofilm [9, 15, 41, 42, 174, 260, 425]. Due to its strong adherence to vaginal epithelial cells and biofilm-forming capacities, it has been suggested that *G. vaginalis* initiates the colonisation of the vaginal epithelium and serves as a scaffolding to which other species subsequently can attach [141, 209, 425].

One of the species that might attach to the biofilm initiated by *G. vaginalis* could be *Atopobium vaginae* [163, 166]. Several molecular studies have indicated a probable role for *A. vaginae* in BV [165, 166, 171], and it has also been suggested that *A. vaginae* plays a major part in the establishment of a biofilm, together with *G. vaginalis* [9, 425]. Considering it has been found in 80-90% of cases of relapse [170] and some strains have been shown in vitro to be metronidazole resistant [330], it could be of importance in the recurrence of BV after standard treatment with metronidazole.

The current gold standard in BV research is the microscopic evaluation and scoring of vaginal slides according to Nugent [158]. The diagnosis of BV is based on the absence of lactobacilli and the presence of small Gram-negative to Gram-variable rods (*G. vaginalis* and Bacteroides spp. morphotypes) and curved Gram-negative rods (Mobiluncus spp. morphotypes). In fact, bacterial biofilm can also be seen with this method, in the form of clue cells, which are vaginal epithelial cells covered by layers of adherent Gram-negative and/or Gram-variable cells, that is, biofilms [426]. Using Gram staining, it is impossible to distinguish between the different bacterial species in the biofilm. By labelling the cells with a fluorescent probe, using fluorescence in situ hybridisation (FISH), the structure and composition of the biofilm can be studied in more detail. To study the potential role of *A. vaginae* and the synergy between *A. vaginae* and *G. vaginalis* in the biofilm, we used our newly developed peptide nucleic acid (PNA) *A. vaginae* probe [209] together with an existing probe for *G. vaginalis* [204] and a universal bacterial probe [416] to investigate the composition of vaginal biofilm and its importance in BV.

4.3.2 Methods and materials

Clinical samples

Vaginal sample collection and preparation Vaginal samples were collected from 120 women participating in a clinical trial at Rinda Ubuzima in Kigali, Rwanda, studying the safety and acceptability of a contraceptive vaginal ring (NuvaRing®), including the effect of the vaginal ring on the vaginal microbiome (the Ring Plus study - Clinicaltrials.gov NCT01796613) [412]. Participants were between 18 and 35 years old and provided written informed consent for participation in the study. The Ring Plus study was approved by the Rwanda National Ethics Committee, Rwanda (Approval number 481/RNEC/2013); and the ethics committees of the Institute of Tropical Medicine (ITM), Belgium (Approval number 864/13); the Antwerp University Hospital, Belgium (Approval number 13/7/85); and the University of Liverpool, UK (Approval number RETG000639IREC).

Depending on the group (continuous or intermittent ring use) to which the participant was randomised, a total of four or five samples from the same participant were taken over a period of four menstrual cycles. A total of 463 samples were analysed after Gram stain and after FISH using light microscopy and confocal laser scanning microscopy (CLSM), respectively. Vaginal sampling was carried out by the study physician during a speculum examination in the Rinda Ubuzima research clinic. A cotton swab was brushed against the lateral walls of the vagina and was transported in its container to the Rinda Ubuzima laboratory within 20 min. Upon arrival in the laboratory, the swab was used to prepare a vaginal slide on a regular glass slide for Gram stain and a second vaginal slide on a Superfrost Plus slide (Menzel-Gläser, Braunschweig, Germany).

All slides were air dried, heat-fixed by passing through a flame twice and then stored in their appropriate boxes until Gram staining and/or shipment for FISH. The first slide was Gram stained and examined on-site in the Rinda Ubuzima laboratory in Kigali. The Superfrost Plus slides were stored and shipped at room temperature to the ITM where they were fixed for a minimum of 12 h in Carnoy solution (6:3:1, ethanol:chloroform:glacial acetic acid) [209].

Microbiological analysis of the vaginal samples

Peptide nucleic acid fluorescence in situ hybridisation PNA FISH was performed as described earlier [209] using species-specific probes for *A. vaginae* (AtoITM1) and *G. vaginalis* (Gard162), and the broad-range BacUni-1 probe. The hybridised samples were stored in the dark at room temperature for a maximum of 1 week before microscopic observation, using CLSM (LSM700, Zeiss, Oberkochen, Germany). Detection and identification of individual bacteria were done at 400X magnification (objective: Plan-Apochromat 40x/1.3 Oil Ph3 M27). Separate scattered bacterial cells were defined as dispersed bacteria. Aggregates of bacterial cells, sticking to the vaginal epithelial cells, were defined as adherent bacteria forming a biofilm. The species-specific signal was considered positive only if it had a positive counterpart in the DAPI stain and if it displayed a positive signal simultaneously with the universal probe. Semi-quantification was done for the dispersed and adherent bacteria in three categories (absent, present in low amount, present in high amount), but for the analysis only two categories (absent or present) have been used.

Nugent score The status of the vaginal microbiome was assessed at the Rinda Ubuzima laboratory by Nugent scoring of a Gram-stained vaginal slide [158]. A score of 0-3 was considered normal vaginal microbiome; a score of 4-6 intermediate microbiome and a score of 7-10 BV.

Statistical analysis

The clinical study sample size calculation was based on the primary objective to assess the pre-post changes in the vaginal microbiome and required 60 women in each group to require 95% power to detect clinically important changes in bacterial counts [412]. Data analysis was done using STATA10 (StataCorp LP, Texas, USA). While the samples were collected longitudinally, they were analysed cross-sectionally, with each sample as the unit of analysis. To study the association between the presence and absence of dispersed and/or adherent *A. vaginae* and adherent *G. vaginalis* in relation to BV status, we categorised the samples into five categories (Table 4.7), based on combinations of the presence of both bacteria in dispersed and/or adherent form as visualised by FISH. To increase the statistical power, we made the vaginal microbiome status binary: Nugent score 0-3 (reference group) versus Nugent score 4-10 (Table 4.8). A mixed-effects logistic regression model was fitted with BV as the binary outcome (ie Nugent 0-3 vs Nugent 4-10) and biofilm characteristics as the main dependent variable. The model was adjusted for woman, randomisation group, and study visit, because multiple samples per woman at multiple study visits were included in the analysis. ORs are reported with 95% CI and the p-values are from X^2 tests (Table 4.8).

4.3.3 Results

Characterisation of vaginal samples

In total, 463 of 527 samples from 120 women were available for FISH analysis, excluding 13 missing samples and 51 samples not readable due to the absence of epithelial cells on the slides. In all 463 samples, a positive signal was detected for the universal BacUni-1 probe. In 230 samples (49.7%), only dispersed bacteria were present, while the other 233 slides (50.3%) contained adherent bacteria as well (Table 4.7). *A. vaginae* and *G. vaginalis* were part of this biofilm in 126 (54.1%) and 191 (82.0%) samples respectively. Next, we visualised *A. vaginae* with FISH in 195 (42.1%) samples; in 69 samples (14.9% of the total 463 samples) *A. vaginae* was present in a dispersed state whereas in 126 samples (27.2%) the *A. vaginae* bacteria were seen adherent to epithelial cells (Table 4.7). For 122 (97.0%) of the samples with adherent *A. vaginae*, concurrent dispersed *A. vaginae* bacteria were observed. *G. vaginalis* was detected by FISH in 291 (62.9%) samples; it was detected as dispersed-only *G. vaginalis* in 100 samples (21.6% of the total 463 samples) and for the remaining 191 samples (41.3%) *G. vaginalis* was adherent to the epithelial cells. Furthermore, when combining the results of both bacteria and considering only the 291 *G. vaginalis* FISH-positive samples, *A. vaginae* was absent in 98 of the slides (33.7%). On the contrary, only two (0.5%) of the 195 samples showing *A. vaginae* (dispersed and/or adherent) with FISH were negative for *G. vaginalis*; this included one sample with adherent *A. vaginae*.

One-third of the vaginal samples (n=156; 33.7%) was classified as Nugent score 7-10, 10% as Nugent score 4-6 (n=48; 10.4%) and the remaining 259 samples (55.9%) as Nugent 0-3. The majority of the samples without *A. vaginae* (n=201; 75.0%) and without *G. vaginalis* (n=155; 90.1%) were categorised as Nugent 0-3, thus indicating a healthy microbiome. A BV microbiome, defined by a Nugent 7-10 category, was present in 75.4% of samples with adherent *A. vaginae* (n=95) and in 69.6% of the slides with adherent *G. vaginalis* (n=133). In case of absent *G. vaginalis* and *A. vaginae* by FISH (n=170, 36.7%), a healthy microbiome (Nugent 0-3) was observed for 90.0% of the 170 samples (n=153). Furthermore, when considering *G. vaginalis* and *A. vaginae* adherent samples only (n=126), 75.4% of the samples were categorised as BV (Nugent 7-10) (FISH experiments in figure 4.6; table 4.7).

The presence of *A. vaginae*, *G. vaginalis* and combinations of both bacteria in dispersed and adherent forms in relation to BV status

The group of FISH samples without *A. vaginae* and *G. vaginalis* was used as the reference group (Table 4.8). Compared with this reference group, the odds of having a Nugent

Figure 4.6: Superimposed confocal laser scanning microscopy images with 400x magnification of *Atopobium vaginae* + *Gardnerella vaginalis* biofilm, in six vaginal samples (A-F): vaginal epithelial cells DAPI in blue, *A. vaginae* specific peptide nucleic acid (PNA)-probe AtoITM1 with Alexa Fluor 488 in green and *G. vaginalis* specific PNA-probe Gard162 with Alexa Fluor 647 in red. For clarity, we omitted the BacUni-1 plane, such that the bacteria that did not hybridise with Gard162 and AtoITM1 are visible in DAPI blue only.

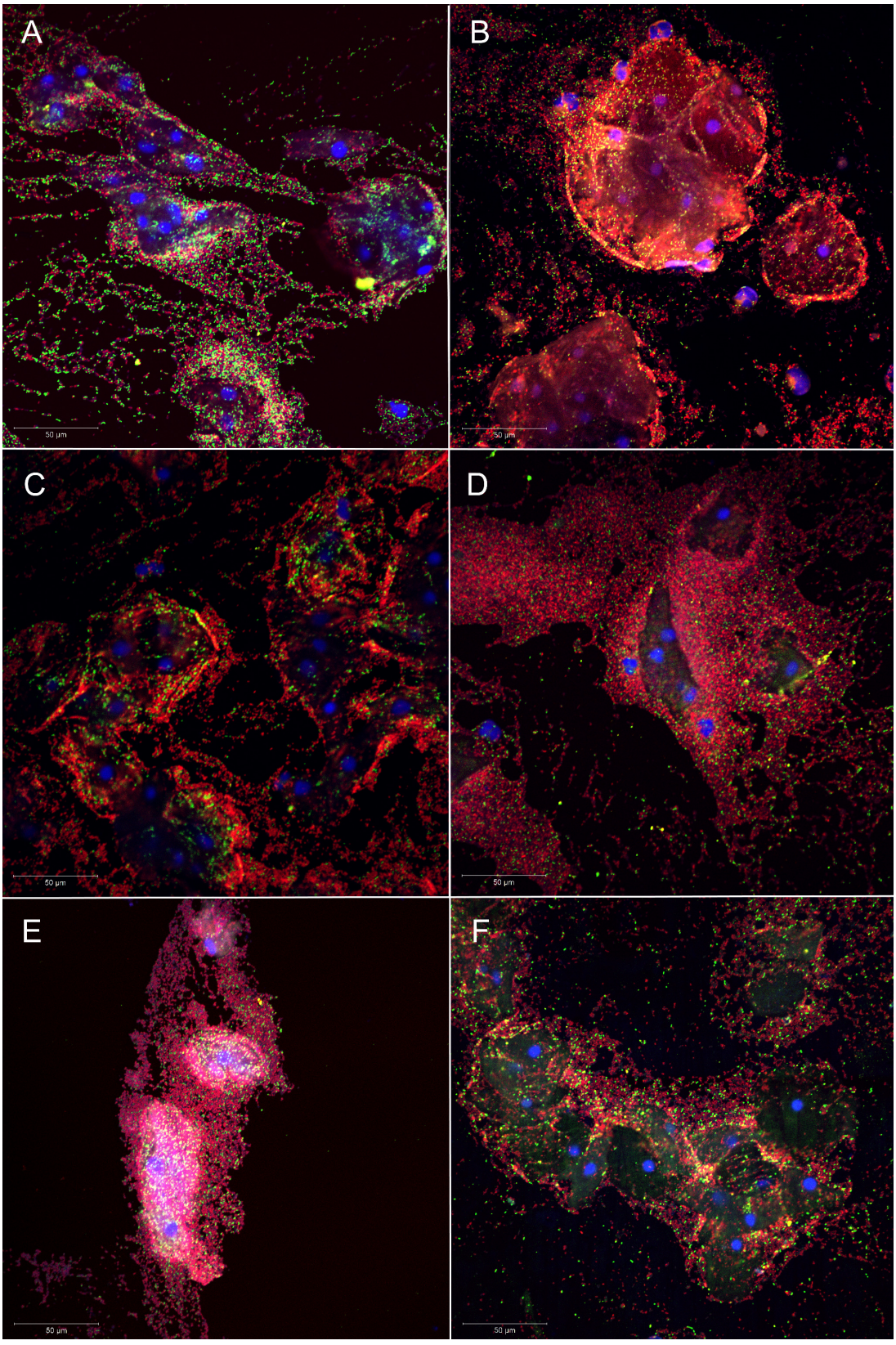


Table 4.7: *Gardnerella vaginalis*, *Atopobium vaginae*, and *G. vaginalis* with *A. vaginae* combinations for samples analysed with fluorescence in situ hybridisation (FISH) by absent, dispersed only, and adherent ± dispersed category and stratified by Nugent scoring

		Nugent 0-3	Nugent 4-6	Nugent 7-10
FISH all bacteria				
absent	0	0 (0.0)	0 (0.0)	0 (0.0)
dispersed only	230	197 (76.0)	19 (39.6)	14 (9.0)
adherent ± dispersed	233	62 (24.0)	29 (60.4)	142 (91.0)
FISH *A. vaginae* **(Av)**				
absent	268	201 (77.6)	24 (50.0)	43 (27.6)
dispersed only	69	41 (15.8)	10 (20.8)	18 (11.5)
adherent ± dispersed	126	17 (6.6)	14 (29.2)	95 (60.9)
FISH *G. vaginalis* **(Gv)**				
absent	172	155 (59.8)	8 (16.7)	9 (5.8)
dispersed only	100	71 (27.4)	15 (31.2)	14 (9.0)
adherent ± dispersed	191	33 (12.8)	25 (52.1)	133 (85.2)
FISH Av and Gv combined				
Gv and Av absent	170	153 (59.1)	8 (16.7)	9 (5.7)
Gv or Av dispersed only	101	72 (27.8)	15 (31.2)	14 (9.0)
Gv adherent ± Gv dispersed and Av absent	51	14 (5.4)	8 (16.7)	29 (18.6)
Gv adherent ± Gv dispersed and Av dispersed	15	3 (1.1)	3 (6.2)	9 (5.8)
Gv and Av adherent ± Gv and Av dispersed	126	17 (6.6)	14 (29.2)	95(60.9)

score of 4-10 were increased when one or both bacteria were present in the dispersed state without adhering to the vaginal epithelium (OR 4.5 (CI: 2 to 10.3)); it was increased further when *G. vaginalis* was part of an adherent biofilm on the epithelium (OR 49.2 (CI: 15.9 to 151.8)) and even more when dispersed *A. vaginae* accompanied this *G. vaginalis* biofilm (OR 75.6 (CI: 13.3 to 429.5)); ultimately the OR was highest when *A. vaginae* was part of the *G. vaginalis* biofilm as well (OR 119 (CI: 39.9 to 360.8)).

Table 4.8: Association between the bacterial presence of *Atopobium vaginae* and *Gardnerella vaginalis* by fluorescence in situ hybridisation (FISH) and the vaginal microbiome defined by Nugent scoring

G. vaginalis (Gv) and *A. vaginae* (Av) combination	absent	Gv or Av dispersed only	Gv adherent ± Gv dispersed and Av absent	Gv adherent ± Gv dispersed and Av dispersed only	Gv and Av adherent ± Gv and Av dispersed
Total = 463	170	101	51	15	126
Nugent 0-3	153 (90.0)	72 (71.3)	14 (27.5)	3 (20.0)	17 (13.5)
Nugent 4-6	17 (10.0)	29 (28.7)	37 (72.5)	12 (80.0)	109 (86.5)
OR (CI)[1]	Reference	4.5 (2 to 10.3)	49.2 (15.9 to 151.8)	75.6 (13.3 to 429.5)	119 (39.9 to 360.8)
p Value X^2 test		0.001	<0.001	<0.001	<0.001

[1] The mixed-effects logistic regression model was adjusted for woman, randomisation group and visit.

4.3.4 Discussion

We set out to study the potential role of *A. vaginae* in BV and the synergy between *A. vaginae* and *G. vaginalis* in the BV-associated biofilm.

Our study confirms that both *A. vaginae* and *G. vaginalis* are important constituents of the vaginal epithelial biofilm [9, 209]. Adherent *A. vaginae* and *G. vaginalis* were visualised in respectively 54.1% and 82.0% of samples with bacterial biofilm (detected using the universal BacUni-1 probe), suggesting an important role for both bacteria in this polymicrobial biofilm. Using FISH, we only found two samples containing *A. vaginae* (dispersed in both, adherent in one) in the absence of *G. vaginalis*, while more than one-third of the *G. vaginalis*-positive samples was negative for *A. vaginae*. This is in accordance with prior reports on the association of *A. vaginae* with *G. vaginalis* [9, 164, 165, 171, 209]. We showed that the presence of both bacteria in the samples, regardless of their existence in a biofilm, was associated with an elevated or high Nugent score, indicative for vaginal dysbiosis and BV. The highest probability of having a Nugent score higher than 3 was seen when both *A. vaginae* and *G. vaginalis* were part of a biofilm attaching to the vaginal epithelial cells.

The association of *G. vaginalis* with BV was originally described in 1954 by Gardner and Dukes [427]. The involvement of *A. vaginae* in BV, however, has only been established 10 years ago [163, 165, 166]. Swidsinski et al. [9] found vaginal biopsies with vaginal biofilm to be positive for *G. vaginalis* and *A. vaginae* when using fluorescent probes, although in our hands this *A. vaginae* probe cross-reacted with other vaginal species as well [209].

The presence of *A. vaginae* in the BV-associated biofilm could have a major impact on treatment. Susceptibility to metronidazole, the standard treatment for BV, varied significantly across various *A. vaginae* strains in vitro [330]. In vivo data are scarce, but Bradshaw et al. [170] found that rates of recurrence of BV were higher when *A. vaginae* was present in the vaginal microbiome in addition to *G. vaginalis*. In another study with topical metronidazole gel by Ferris et al. [163], it was shown that a high concentration of *A. vaginae* before treatment was associated with complete or partial failure of treatment for BV. In the above studies, no distinction was made between dispersed and biofilm-associated bacteria. Nevertheless, as bacteria in a biofilm are less sensitive to antibiotic treatment [428] and considering the evidence from our study that the formation of a bacterial biofilm is more likely to occur when *A. vaginae* is present in the vaginal microbiome, future design of studies may want to take this distinction into account when treating BV.

Our study has shed new light on the significance of *A. vaginae* and the synergy between *A. vaginae* and *G. vaginalis* in vaginal dysbiosis, using highly specific PNA probes for both bacteria. However, a limitation was that we used multiple samples from the 120 women of the Ring Plus study. Ideally, we should repeat the study in a larger group of women. Furthermore, although we assessed the association between bacterial biofilm and vaginal dysbiosis, more research is needed to unravel the exact mechanisms of biofilm formation in BV, including the role and the importance of both bacteria studied, to finally define improved regimens for treatment of BV. Moreover, since BV is a polymicrobial condition,

new research should study the involvement of other bacteria related to BV.

In conclusion, the presented study uncovered a key piece of the BV puzzle confirming first, the importance of *A. vaginae* in BV-associated biofilm and second, showing the joint presence of *A. vaginae* and *G. vaginalis* in a biofilm. Future studies covering a wide array of BV-associated bacteria may help to further delineate biofilm mechanisms in BV.

4.4 The importance of *Gardnerella vaginalis* sialidase for bacterial vaginosis-associated biofilm

Abstract

Bacterial vaginosis is adifficult-to-treat recurrent condition in which health-associated lactobacilli are outnumbered by other anaerobic bacteria, such as *Gardnerella vaginalis*. Certain genotypes of *G. vaginalis* can produce sialidase, while others can not. Sialidase facilitates the destruction of the protective mucus layer on the vaginal epithelium by hydrolysis of sialic acid on the glycans of mucous membranes. This process facilitates adhesion of bacterial cells on the epithelium and has been linked with the development of biofilm in other microorganisms. In this study, using vaginal specimens of 120 women enrolled in the Ring Plus study, we assessed the association between the *G. vaginalis* sialidase gene by quantitative polymerase chain reaction, the diagnosis of bacterial vaginosis according to Nugent score, and the occurrence of a bacterial vaginosis-associated biofilm dominated by *G. vaginalis* by fluorescence in situ hybridisation. We detected the sialidase gene in 75% of the *G. vaginalis*-positive samples and found a strong association ($p<0.001$) between the presence of a *G. vaginalis*-dominated biofilm, the diagnosis of BV according to Nugent and the detection of high loads of the *G. vaginalis* sialidase gene in the vaginal specimens. These results could impact the possibilities for diagnosis of bacterial vaginosis, and in addition might guide research for new treatment.

Adapted from:

Hardy L, Jespers V, Van den Bulck M, Buyze J, Mwambarangwe L, Musengamana V, Vaneechoutte M, Crucitti T. The importance of *Gardnerella vaginalis* sialidase for bacterial vaginosis-associated biofilm. Submitted for publication.

4.4.1 Introduction

Gardnerella vaginalis has consistently been found in bacterial vaginosis (BV) [15, 40, 41], a dysbiosis of the vaginal econiche in which the health-associated lactobacilli are outnumbered by other micro-aerophilic and anaerobic organisms. It has been demonstrated that a vaginal mucosa polymicrobial biofilm is associated with BV [9, 209]. *G. vaginalis* is able to adhere to the vaginal epithelial cells and subsequently develop a biofilm on the vaginal wall [9, 209], a mechanism that possibly increases the tolerance of *G. vaginalis* to lactic acid and hydrogen peroxide produced by lactobacilli [260] and to antimicrobial treatment [266, 267]. It has been suggested that *G. vaginalis* initiates the colonisation of the vaginal mucosa and acts as a scaffold to which other species subsequently can attach [260, 264, 414]. However, *G. vaginalis* can also occur in the healthy vaginal microbiome (although in lower concentrations) [3, 40, 41], suggesting that the mere presence of *G. vaginalis* does not necessarily result in biofilm formation and BV. This observation has led several researchers to hypothesise that different types of *G. vaginalis* with different virulence potentials might exist [160, 162, 429].

Certain *G. vaginalis* genotypes can produce sialidase, also known as neuraminidase [160]. Sialidase is a common virulence factor in pathogens such as the Influenza virus [285] and a large number of bacterial species, such as *Propionibacterium acnes* [286], *Pseudomonas aeruginosa* [287], *Streptococcus pneumoniae* [288], and *Vibrio cholerae* [289] and has been strongly linked with bacterial biofilm development [290–292]. Increased sialidase activity was also detected in the vaginal fluid of BV patients [293, 430], and is the basis of a marketed quick test for diagnosis of BV [294].

Sialidase facilitates the destruction of the protective mucus layer in the vagina by hydrolysis of sialic acid, the most distal sugar moiety on the glycans of mucous epithelial membranes [293, 295]. Cleaving off sialic acid by sialidase provides the bacteria with free sialic acid that can serve as a nutrient [296], and the exposure of the underlying glycan-binding site facilitates adhesion of bacterial cells [295, 431]. *G. vaginalis* could benefit from this mechanism by attaching to the vaginal epithelium to initiate biofilm development. Furthermore, sialidase facilitates the circumvention of the vaginal adaptive immune response, since it modulates the activity of sialylated immune mediators such as interleukins, immunoglobulins and various cellular receptors, such as sialic acid lectins (Siglecs) [297]. In addition, it has been suggested that, by incorporation of the cleaved sialic acids into bacterial cell-surface structures, bacteria could disguise themselves as host cells and bypass the host's immune response [431, 432].

Although other BV-associated bacteria (e.g. *Prevotella* and *Bacteroides* species) have also been shown to produce sialidase in the vagina [293], *G. vaginalis* is most frequently isolated, in high concentrations, from vaginal fluid of women with BV [15, 40, 41] and has

a higher tendency to adhere to vaginal epithelial cells compared to other BV-associated anaerobes [260]. We hypothesised that, like other species [290–292], the genotypes of *G. vaginalis* that encode the sialidase gene are associated with the presence of vaginal biofilms, leading to BV. Therefore, we assessed the association between the presence of the *G. vaginalis* sialidase gene and the occurrence of BV-associated biofilm of *G. vaginalis* on the vaginal epithelium in vaginal samples of women with and without BV.

Methods and materials

Study participants and ethics statement Vaginal samples were collected from 120 Rwandan women participating in study on the acceptability of using an intravaginal ring for contraception (NuvaRing®, Merck, New Jersey, USA) and its effect on the vaginal microbiome (the Ring Plus study [412]). Participants were between 18 and 35 years old and provided written informed consent for participation in the study. The Ring Plus study was approved by the Rwanda National Ethics Committee, Rwanda (Approval number 481/RNEC/2013); and the ethics committees of the Institute of Tropical Medicine (ITM), Belgium (Approval number 864/13); the Antwerp University Hospital, Belgium (Approval number 13/7/85); and the University of Liverpool, UK (Approval number RETG000639IREC).

Vaginal sample collection and preparation

Vaginal samples were collected at the enrolment visit and at each ring insertion visit by the study clinician. Two Copan flocked® swabs (Copan, Brescia, Italy) and one cotton swab were brushed against the lateral walls of the vagina. The cotton swab was immediately used to prepare two vaginal slides: one for Gram staining and one for fluorescence in situ hybridisation (FISH) on a Superfrost Plus® slide (Menzel-Gläser, Braunschweig, Germany). Both were heat-fixed by passing twice through a flame. The Superfrost Plus slides were stored and shipped at room temperature to the ITM, to be fixed for a second time using Carnoy solution (6:3:1, ethanol:chloroform:glacial acetic acid) [209] for 12 hours minimum. The Copan flocked swabs were eluted by vortexing for at least 15 seconds in 1.2 ml of diluted phosphate buffered saline (PBS) (pH 7.4 - 1:9, PBS:saline). The eluates were stored at -80 °C until shipment and shipped to the ITM using a temperature-controlled dry shipper.

Nugent score of vaginal slides

The status of the vaginal microbiome was assessed at the Rinda Ubuzima laboratory (Kigali, Rwanda) by Nugent scoring of a Gram stained vaginal slide [158]. A score of

0-3 was considered as normal vaginal microbiome; a score of 4-6 as intermediate vaginal microbiome and a score of 7-10 as BV.

Peptide nucleid acid fluorescence in situ hybridisation on vaginal slides

Peptide nucleid acid (PNA) fluorescence in situ hybridisation (FISH) on the vaginal slides using a species-specific probe for *G. vaginalis* (Gard162) and the broad-range BacUni-1 probe and imaging was performed as described earlier [209]. Separate scattered bacterial cells were defined as dispersed bacteria. Aggregates of bacterial cells, sticking to the vaginal epithelial cells, were defined as adherent bacteria forming a biofilm.

4.4.2 Quantitative polymerase chain reaction for quantification of bacteria in vaginal samples

Quantitative polymerase chain reaction for *Gardnerella vaginalis*

The total bacterial load of *G. vaginalis* was determined by means of quantitative polymerase chain reaction (qPCR) in the Sexually Transmitted Infections (STI) Reference Laboratory at the ITM, as described before [3, 209]. The bacterial load was expressed as genome equivalents (geq)/ml.

Quantitative polymerase chain reaction for *Gardnerella vaginalis* sialidase The design of the primer set for amplification of the *G. vaginalis* sialidase gene was based on previous work by Lopes dos Santos Santiago et al. [160] and on the sequence of sialidase A from the fully sequenced *G. vaginalis* ATCC 14019 strain (reference genome for the Human Microbiome Project, Baylor College of Medicine, Houston, TX). The previously designed *G. vaginalis* sialidase forward primer (GVSI_Forward, 5'-GACGACGGCGAATGGCACGA-3') [160] was combined with a reverse primer (GVSI_Reverse2, 5'-TACAAGCGGCTTTACTC-TTG-3') that was newly designed using Primer Blast (National Center for Biotechnology Information, Bethesda, MD).

For *G. vaginalis* sialidase gene amplification, the 25 µl PCR mixture contained 12.5 µl Rotor-Gene SYBR Green qPCR Master mix (Qiagen, Venlo, the Netherlands), 5 µl DNA extract, 0.75 µM of 5 µM *G. vaginalis* sialidase forward and reverse primers (Integrated DNA Technologies, Leuven, Belgium) and RNase-free water provided with the Rotor-Gene SYBR Green PCR kit.

The amplification reactions were performed using the Rotor Gene Q MDx 5 plex (Qiagen, Venlo, the Netherlands) and the amplification program (10 min 95 °C, (5 sec 95 °C - 10

sec 58 °C) x 45) was followed by melting curve analysis. Each sample was run in duplicate and each run included a standard curve.

Statistical analysis

Bacterial counts were log 10 transformed before analysis. Data analysis was done using STATA13. The p-values reported for associations between the presence and quantity of the sialidase gene and BV/qPCR-biofilm results were obtained using mixed effects ordered logistic regression.

4.4.3 Results

Characterisation of vaginal samples

A total of 527 samples were available for Nugent scoring, 462 samples were analysed by FISH and 524 samples were used for qPCR to detect *G. vaginalis*. All 393 *G. vaginalis* qPCR-positive samples were tested for the presence of the *G. vaginalis* sialidase gene by qPCR (Table 4.9).

Table 4.9: Characteristics of vaginal samples

Test	Total	Result	N (%)
Nugent score	527	0-3	299 (56.7)
		4-6	53 (10.1)
		7-10	175 (33.2)
FISH	462	*Gardnerella vaginalis* positive	290 (62.8)
		G. vaginalis biofilm	191 (41.3)
		G. vaginalis dispersed only	99 (21.4)
qPCR	524	*G. vaginalis* positive	393 (75.0)
		G. vaginalis sialidase positive	294 (56.1)

Nugent score Of the total of 527 samples of 120 participants, 299 (56.7%) had a healthy microbiome (Nugent score 0-3), 53 (10.1%) were categorised as intermediate (Nugent score 4-6) and 175 (33.2%) were diagnosed as BV (Nugent score 7-10).

Fluorescence in situ hybridisation A subset of 462 samples were analysed with FISH. The remaining 65 samples could not be analysed mainly due to the absence of epithelial cells and bacteria on the slides. *G. vaginalis* was present in 290 samples (62.8%) using

FISH. In 191 of these *G. vaginalis* positive samples (65.9%), the bacteria were attached to the vaginal epithelium and considered to be part of a biofilm, although dispersed bacteria were present as well (Figure 4.7). In the other 99 samples (34.1%), *G. vaginalis* was only present in the dispersed form.

Quantitative polymerase chain reaction A total of 524 samples were available for quantification of *G. vaginalis*. *G. vaginalis* was detected in 393 samples (75.0%), with a mean bacterial load (log 10) of 6.97 ± 1.37 (standard deviation) geq/ml. Moreover, the presence of the *G. vaginalis* sialidase gene was assessed in all 393 *G. vaginalis*-qPCR positive samples and was present in 294 samples (74.8%). The *G. vaginalis* sialidase gene was detected with $<10^6$ geq/ml (low load) in 112 samples (28.5%) and with $>10^6$ geq/ml (high load) in the remaining 182 samples (46.3%).

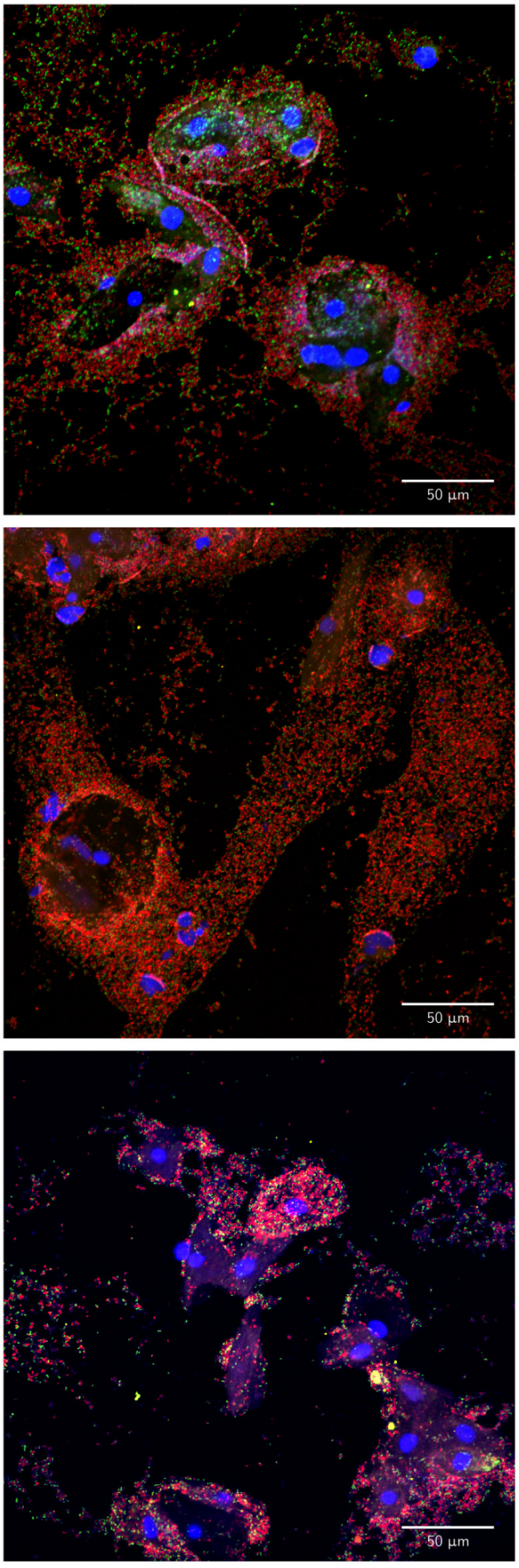
Association between the presence of the sialidase gene and biofilm

Both FISH and *G. vaginalis* sialidase qPCR analysis were carried out for a subset of 345 samples. Based on our data, the presence of the *G. vaginalis* sialidase gene, as assessed by qPCR, is associated with the presence of *G. vaginalis* biofilm, as assessed with FISH ($p<0.001$) (Table 4.10). For the 163 samples with a high load of *G. vaginalis* sialidase (i.e. $>10^6$ geq/ml), *G. vaginalis* biofilm was present in 75.5%, whereas dispersed-only *G. vaginalis* was present in only 14.7% of these samples. *G. vaginalis* was completely absent in only 9.8% of these samples. In the 96 samples with a *G. vaginalis* sialidase low load ($<10^6$ geq/ml), an equal distribution between the three categories was observed; with 36.5% FISH-positive samples with visible biofilm, 29.2% FISH-positive samples with only dispersed *G. vaginalis* and 34.4% *G. vaginalis* FISH-negative samples. Out of the 86 samples that were negative for the *G. vaginalis* sialidase qPCR, *G. vaginalis* biofilm could be detected by FISH in only 17.4% of the samples, while 27.9% contained only dispersed *G. vaginalis* and 54.7% were FISH-negative.

Table 4.10: The association between fluorescence in situ hybridisation, Nugent score and sialidase quantitative polymerase chain reaction results of vaginal samples

	Gardnerella vaginalis sialidase 0 N (%)	*G. vaginalis* sialidase >0 and $<10^6$ N (%)	*G. vaginalis* sialidase $>10^6$ N (%)	P-value
FISH *G. vaginalis*	86 (100)	96 (100)	163 (100)	<0.001
G. vaginalis absent	47 (54.7)	33 (34.4)	16 (9.8)	
G. vaginalis dispersed only	24 (27.9)	28 (29.2)	24 (14.7)	
G. vaginalis biofilm	15 (17.4)	35 (36.5)	123 (75.5)	
Nugent score	98 (100)	112 (100)	181(100)	<0.001
Nugent 0-3	80 (81.6)	61 (54.5)	37 (20.4)	
Nugent 4-6	8 (8.2)	14 (12.5)	28 (15.5)	
Nugent 7-10	10 (10.2)	37 (33.0)	116 (64.1)	

Figure 4.7: Superimposed confocal laser scanning microscopy images with 400x magnification of *Atopobium vaginae* + *Gardnerella vaginalis* biofilm, in three vaginal samples: vaginal epithelial cells DAPI in blue, *A. vaginae* specific peptide nucleic acid (PNA)-probe AtoITM1 with Alexa Fluor 488 in green and *G. vaginalis* specific PNA-probe Gard162 with Alexa Fluor 647 in red.



Association between the presence of the sialidase gene and the diagnosis of bacterial vaginosis

A subset of 391 samples was available for both Nugent scoring and qPCR based on the quantification of the *G. vaginalis* sialidase analysis. The probability of having BV according to Nugent is increased when the *G. vaginalis* sialidase gene is present in high loads (>10^6 geq/ml) (p<0.001) (Table 4.10). Of the 181 samples with a high load of the *G. vaginalis* sialidase gene, 64.1% were BV-positive (Nugent score 7-10), while only 20.4% had a healthy vaginal microbiome (Nugent score of 0-3) and 15.5% were diagnosed with an intermediate Nugent score of 4-6. In contrast, BV could be diagnosed in only 10 out of the 98 samples (10.2%) for which no *G. vaginalis* sialidase gene could be detected. However, 80 samples (81.6%) without the sialidase gene represented a healthy vaginal microbiome according to Nugent and 8 samples (8.2%) had an intermediate score. Additionally, when the *G. vaginalis* sialidase gene was present in low amounts, 54.5% of the 112 samples were considered healthy according to the Nugent score, 33.0% were categorised as BV, and 12.5% represented an intermediate microbiome.

4.4.4 Discussion

BV is the most prevalent vaginal disorder in women of reproductive age worldwide, and aside from the discomfort in case of symptomatic BV, it can also generate an array of serious gynaecological and obstetric complications. The presence of BV-associated anaerobes in the vaginal environment increases the risk for preterm labor and birth [107]. Furthermore, the presence of sialidase in vaginal fluid has been linked to BV and to preterm birth as well [111, 112]. In a large cohort of 1806 women which included 800 women with BV and 53 spontaneous preterm births, Cauci et al. [112] showed that the sialidase levels in the vaginal fluid were significantly associated with all averse pregnancy outcomes.

G. vaginalis plays an important role in BV, since *G. vaginalis* overgrowth is found in nearly all cases of BV [157]. However, the presence of *G. vaginalis* in healthy vaginal environments [3, 433] contradicts its pathogenic role in BV. To resolve this discrepancy, it has been suggested that *G. vaginalis* might actually consist of different species with distinct roles in BV pathogenesis, which is supported by the genotypic and phenotypic diversity of the species [159, 161, 162]. Although other BV-associated bacteria (e.g. *Prevotella* and *Bacteroides* species) are able to produce sialidase [293], we decided to investigate *G. vaginalis* sialidase in BV, considering that *G. vaginalis* is most frequently isolated from vaginal fluids of women suffering from BV [15, 40, 41, 157] and that it has a higher tendency to adhere to vaginal epithelial cells in vitro compared to other BV-associated anaerobes [260]. We studied the association between the presence of the *G. vaginalis*

sialidase gene, as a proxy for sialidase production, in the vagina and the occurrence of BV and bacterial biofilm on the vaginal epithelium. To this end, we screened the vaginal samples of 120 Rwandan women [412] by means of a *G. vaginalis* sialidase specific qPCR and assessed the occurrence of BV and biofilm by means of a) light microscopy after Gram staining and Nugent scoring and b) CLSM after FISH for *G. vaginalis* and bacteria in general on vaginal samples.

In this population, the sialidase gene was detected in about 75% of the *G. vaginalis*-positive samples. In about 60% of those samples a high load ($>10^6$ geq/ml) of the gene was detected. This high prevalence of the *G. vaginalis* sialidase gene in our study may be explained by the cohort of women enrolled for this study and the high prevalence of BV, i.e. in 43.3% of all samples. Earlier studies have investigated the presence of the *G. vaginalis* sialidase gene and the production of sialidase in cultured isolates. Using clinical isolates from Belgian women, Lopes dos Santos Santiago and colleagues could detect a *G. vaginalis* sialidase gene with qPCR in 51% of strains [160]. When using the filter paper spot test for the detection of sialidase activity, von Nicolai et al. [434] could detect sialidase production in only 1 of 10 clinical isolates. Additionally, Briselden et al. [293] detected sialidase activity in 20% of 105 *G. vaginalis* isolates (with no difference in isolates from women with and without BV), and Moncla and Pryke [435] observed sialidase activity in 39% of 31 isolates.

At present, it is not clear whether the sialidase gene is expressed constitutively or not. Pleckaityte and colleagues [436] detected a sialidase gene in 17 tested *G. vaginalis* isolates, but only 10 of these strains actually produced sialidase in vitro. Schellenberg et al. [159] also found that the gene presence was not predictive of actual sialidase activity using a qualitative (positive/negative) filter spot assay: out of 77 *G. vaginalis* isolates positive for the sialidase gene, 36 produced sialidase [159]. In addition, in currently ongoing (not yet published) in vitro experiments by our group, we found that only 29 out of 41 sialidase gene-positive *G. vaginalis* isolates produced sialidase, based on the filter spot test. Interestingly, we noticed that all but two sialidase-producing strains were isolated from women with BV according to Nugent. This contradicts what was published by Lopes dos Santos Santiago et al. [160] who found a 100% correspondence between the mere presence of the gene and sialidase activity in 19 *G. vaginalis* isolates. However, all but one of these isolates were obtained from women with a disturbed microbiome, which might have introduced a bias [160]. The absence of sialidase activity in sialidase-positive isolates might be explained by the presence of an alternative gene encoding this activity or the need for other factors to stimulate the expression of the gene. Possible factors interfering with the production of sialidase might be the presence of sialic acid on epithelial cells, or a threshold in *G. vaginalis* concentration that needs to be reached. In any case, more basic research is needed to fully understand the sialidase expression pathway.

When looking at the association between the presence of the *G. vaginalis* sialidase gene

and the diagnosis of BV by Nugent score, we found that the probability for having BV (Nugent score 7-10) was increased when a high concentration of the *G. vaginalis* sialidase gene was present in the vaginal samples. This was expected, since sialidase production by *G. vaginalis* is recognised as a virulence factor [436], and has already been associated with BV [112]. In our previous work [209], we confirmed the importance of *G. vaginalis* in the development of a biofilm on the vaginal epithelium in BV, as established by Swidsinski et al. [9] in 2005. Ours was the first study to use clinical samples to demonstrate the significance of *G. vaginalis'* ability to produce sialidase and to document its association with BV and vaginal biofilm. We established a strong association between a high load of the *G. vaginalis* sialidase gene, as measured by qPCR using a specific primer set targeting the *G. vaginalis* sialidase gene, and *G. vaginalis* being part of a vaginal epithelium biofilm, visualised by CLSM after FISH. Sialidase has been linked with biofilm development in other microorganisms. In *Pseudomonas aeruginosa*, sialidase (or neuraminidase) contributes to the initial colonisation of the airway, and colonisation could be blocked in vitro by viral neuraminidase inhibitors [290]. Likewise in pneumococcal infections, sialidase is involved in biofilm formation and pathogenesis of respiratory tract infections [291, 292]. Also, sialidase producing *Propionibacterium acnes* isolates were more associated with acne than sialidase negative isolates [437].

A shortcoming of this study is the absence of isolates. Clinical isolates would have provided valuable information on ARDRA genotyping and the actual sialidase activity. Being able to assess sialidase production by *G. vaginalis* directly in our Ring Plus samples would have been interesting, but since sialidase activity in our mixed samples could also have resulted from other vaginal species, it would have confounded the results. Despite this limitation, we were able to establish that the ability of *G. vaginalis* to produce sialidase is linked to the presence of BV and the existence of a vaginal biofilm. This finding may impact the possibilities for BV diagnosis, but it may also guide future research for new and better treatments for this recurrent and difficult-to-treat condition.

4.5 Association of vaginal dysbiosis and biofilm with contraceptive vaginal ring biomass in African women

Abstract

We investigated the presence, density and bacterial composition of contraceptive vaginal ring biomass and its association with the vaginal microbiome. Of 415 rings worn by 120 Rwandese women for three weeks, the biomass density was assessed with crystal violet and the bacterial composition of biomass eluates was assessed with quantitative polymerase chain reaction (qPCR). The biomass was visualised after fluorescence in situ hybridisation (FISH) and with scanning electron microscopy (SEM). The vaginal microbiome was assessed with Nugent scoring and vaginal biofilm was visualised after FISH. All vaginal rings were covered with biomass (mean optical density (OD) of 3.35; standard deviation (SD) 0.64). Lactobacilli were present on 93% of the rings, *G. vaginalis* on 57%, and *A. vaginae* on 37%. The ring biomass density was associated with the concentration of *A. vaginae* (OD+0.03; 95% confidence interval (CI) 0.01-0.05 for one log increase; p=0.002) and of *G. vaginalis* (OD+0.03; (95% CI 0.01-0.05; p=0.013). The density also correlated with Nugent score: rings worn by women with a BV Nugent score (OD+0.26), and intermediate score (OD+0.09) had a denser biomass compared to rings worn by participants with a normal score (p=0.002). Furthermore, presence of vaginal biofilm containing *G. vaginalis* (p=0.001) and *A. vaginae* (p=0.006) correlated with a denser ring biomass (OD +0.24 and +0.22 respectively). With SEM we observed either a loose network of elongated bacteria or a dense biofilm. In summary, we found a correlation between vaginal dysbiosis and the density and composition of the ring biomass, and further research is needed to determine if these relationships are causal. As multipurpose vaginal rings to prevent pregnancy, HIV, and other sexually transmitted diseases are being developed, the potential impact of ring biomass on the vaginal microbiota and the release of active products should be researched in depth.

Adapted from:

Hardy L, Jespers J, De Baetselier I, Mwambarangwe L, Musengamana V, van de Wijgert J, Crucitti T. Association of vaginal dysbiosis and biofilm with contraceptive vaginal ring biomass in African women. Submitted for publication.

4.5.1 Introduction

Contraceptive vaginal rings are available in high income countries and Latin America but not in sub-Saharan Africa [438]. However, vaginal rings are expected to be introduced in this part of the world in the near future. Most notably, multi-purpose vaginal rings are being developed for the controlled release of drugs to prevent reproductive tract infections, such as HIV (dapivirine ring) [439], herpes simplex virus type 2 (HSV-2) [440], bacterial vaginosis (BV), and pregnancy [441].

Early contraceptive ring studies demonstrated that ring use did not negatively affect the naturally protective vaginal environment including the presence of lactobacilli [68, 442, 443]. Recent more in-depth work showed an increase in healthy bacteria or lactobacilli concentrations with ring use [69, 70, 72]. This effect was thought to be caused by ethinyl estradiol in the ring [69, 70, 72]. Lactobacilli play an important role in the two main states of the vaginal microbiome: the health-associated vaginal microbiome dominated by lactobacilli, and a BV-associated microbiome characterised by a polymicrobial dysbiosis. In dysbiosis, the lactobacilli disappear and the concentrations of facultative anaerobic bacteria, such as *Gardnerella vaginalis* and *Atopobium vaginae*, increase [15]. The anaerobic bacteria will often form a vaginal biofilm [9, 141, 209]. Bacterial biofilms are also known to develop on indwelling medical devices whenever microorganisms find a surface to attach to [407]. The potential development of bacterial biofilm on vaginal rings in vivo has yet to be explored in humans.

We hypothesised that a biomass would develop on vaginal rings, and that rings worn by women with BV-associated dysbiosis would have higher biomass density than rings worn by women with dysbiosis. To investigate this hypothesis, we studied the presence, density and bacterial composition of the biomass on contraceptive vaginal rings and investigated the association between ring biomass density and the vaginal microbiome.

4.5.2 Methods and materials

This is a laboratory sub-study of the "Ring Plus" contraceptive vaginal ring study performed at the Rinda Ubuzima (RU) research clinic in Kigali, Rwanda (ClinicalTrials.gov identifier NCT01796613) [412].

Participants, study product, and clinical sample preparation

The NuvaRing® contraceptive vaginal ring (N.V. Organon, Oss, the Netherlands) was used over a period of three months by 120 adult female participants [412]. The women had each ring inserted for three weeks continuously followed by one week off (intermittent

use) or continuously with no breaks in between the removal of the old/insertion of the new ring every three weeks (continuous use). In the intermittent use group, women used three rings each, while the women in the continuous group used four rings each during the whole study period. Vaginal examination, ring removal, and sample collection were carried out by the study clinician, as described previously [444]. For this sub-study, vaginal fluid was rolled on two microscopy slides and airdried for each participant at baseline and at each ring removal visit. One slide was Gram stained for Nugent scoring, and the other slide was used to assess the presence of a vaginal biofilm.

All rings worn by study participants were collected after removal at study visits. Each ring was cut in three equal parts immediately after removal. The part for the biomass density assessment with crystal violet was submerged in 3 ml of glutaraldehyde for two weeks, transferred to 3 ml of formaldehyde, and stored at 2-8 °C until testing. This part was also used for electron microscopy after the crystal violet assay had been completed. The part for qPCR was stored in diluted phosphate buffered saline (dPBS) (pH 7.4 - 1:9, PBS:saline) at -20 °C. The final part for fluorescence microscopy was stored in Carnoy solution (6:3:1, ethanol:chloroform:glacial acetic acid) at 2-8 °C until testing [209]. All samples, except for the Nugent slide, were shipped from the study site in Rwanda to the Institute of Tropical Medicine (ITM) in Antwerp, Belgium: the refrigerated samples were transported at room temperature, while the frozen samples were transported at -196 °C in a dry shipper.

Laboratory assessment of clinical samples

For this sub-study, all vaginal rings removed and collected throughout the study for both the intermittent and continuous use groups were analysed as well as all vaginal smears collected at baseline and each ring removal visit. The vaginal microbiota were characterised in two ways: Nugent scoring of Gram stained vaginal smears [158] in the on-site RU laboratory and confocal laser scanning microscopy (CSLM) after peptide nucleic acid (PNA) fluorescence in situ hybridisation (FISH) of a second vaginal smear to detect vaginal biofilm at the ITM in Antwerp. In Nugent scoring, a score of 0-3 is considered a normal vaginal microbiota; a score of 4-6 an intermediate microbiota and a score of 7-10 bacterial vaginosis. Vaginal biofilms on another vaginal smear were visualised with CSLM after FISH. This technique was performed as described previously using three probes: the broad-range BacUni-1 probe detecting all bacteria, and probes detecting two bacterial species strongly associated with bacterial vaginosis (AtoITM1 for *A. vaginae* and Gard162 for *G. vaginalis*) [209, 444].

The biomass on contraceptive rings that had been used by participants for three weeks was stained with crystal violet to determine the optical density (OD) as a proxy for the

quantity of the biomass. The crystal violet microtitre plate assay [445] was adapted to fit the ring parts and applied to identify and measure the biomass density on the rings. First, the ring biomass was stained with crystal violet (0.1% solution) for 10 minutes. Next, the ring part was rinsed twice and air-dried and the ring biomass staining was solubilised by submerging the ring in 3 ml of 30% acetic acid in water. From this solution, 125 µl was transferred to a new microtitre plate for OD measurement at 550 nm. Bacterial compositions of the biomass were assessed by quantitative polymerase chain reaction (qPCR) of the *Lactobacillus* genus, *G. vaginalis*, and *A. vaginae*. Frozen ring parts were thawed and vortexed; using this eluate, 200 µl DNA was extracted (Abbott, Maidenhead, UK) and stored at -80 °C until testing. qPCR was performed for each bacteria genus or species separately. The PCR mixtures and primers for *A. vaginae*, *G. vaginalis*, and *Lactobacillus* genus and the amplification reactions (Rotor Gene Q MDx 5 plex, Qiagen, Venlo, the Netherlands) have been described before [209].

In a random sub-sample of 120 rings, bacterial compositions of the biomass were also visualised by CSLM after PNA FISH for *G. vaginalis*, *A. vaginae*, and *Lactobacillus* genus. Slides were prepared for fluorescence microscopy by rinsing the ring with ddH_2O, removing the biomass attached to the ring and spreading it out on the microscopy slide, passing the slide through a flame twice, and fixating it in Carnoy solution. PNA FISH was performed as described earlier [3,444]. An additional probe targeting the *Lactobacillus* genus (Lac663) [204] was used to visualise the *Lactobacillus* species in the biomass. Furthermore, we applied scanning electron microscopy on a random selection of 11 rings to enable a three-dimensional view of the biomass architecture. The ring parts were dehydrated in an ethanol line and critical point dried. The parts were cut in pieces of one centimetre each, mounted on metal specimen stubs, coated with a 16 nm thick platinum film, and imaged using a JEOL JSM-840 microscope.

Statistical analysis

STATA version 12 was used to analyse data. The numbers and proportions of vaginal slides by Nugent score category (0-3, 4-6, 7-10) and fluorescence microscopy evaluation categories were described. Fluorescence microscopy was conducted by one microscopist (LH) who recorded for each vaginal slide and ring part whether she visualised any bacterial biofilm (positive fluorescence signal for the "all bacteria" probe), a biofilm incorporating *G. vaginalis* and/or *A. vaginae* (positive fluorescence signals for the relevant species-specific probe), and/or whether she visualised any of these as dispersed/planktonic bacteria only. Biofilm was defined as a dense network of bacteria adhering to a surface (the vaginal epithelial cells), dispersed/planktonic bacteria were defined as scattered bacteria, not visibly adhering to other bacteria or a surface.

These results were presented in three ways. First, visualisation of any vaginal biofilm ("all bacteria" probe positive), of a biofilm containing *G. vaginalis* and/or *A. vaginae* (each of these was assessed for each slide and results are not mutually exclusive). Second, no biofilm visualised at all, but dispersed/planktonic bacteria were present (for all bacteria, *G. vaginalis*, and *A. vaginae*; not mutually exclusive). Three, no bacteria visualised at all (for all bacteria, *G. vaginalis*, and *A. vaginae*; not mutually exclusive).

Ring biomass density was presented as mean OD with standard deviations; the presence of *Lactobacillus* genus, *G. vaginalis*, and *A. vaginae* in ring biomass eluates as proportions with 95% confidence intervals (CI). The bacterial concentrations of *Lactobacillus* genus, *G. vaginalis*, and *A. vaginae* in ring biomass eluates were expressed as $\log_{10}$ transformed genome equivalents (geq)/ml. We used mixed effects regression analysis with fixed effects for participant (due to repeated observations) and randomisation group (intermittent versus continuous ring use) to evaluate associations between ring biomass density and Nugent score categories, and with fluorescence microscopy results (presence of *G. vaginalis* vaginal biofilm, *A. vaginae* biofilm, *G. vaginalis* in a dispersed form only, and *A. vaginae* in a dispersed form only).

4.5.3 Results

The mean age of the 120 randomised participants was 28.4 years (95% CI: 25-32), with 61% of women being married, and 57.5% having attained more than just primary school education. All participants but one completed the study, which implies that 417 rings and matching vaginal smear duplicates (3 times 60, or 180 from the intermittent group; 4 times 60, or 237 from the continuous group, excluding 3 samples of one discontinued participant) should have been collected. We were able to collect a total of 415 vaginal rings and 415 vaginal smears in duplicate. Two sample sets did not reach the laboratories. A total of 415 vaginal rings were assessed for biomass with the crystal violet assay, 412 ring eluates by qPCR, and sub-samples of 120 rings by FISH and 11 rings with SEM. The quality of 7 slides was insufficient for Nugent scoring, leaving us with 408 vaginal slides to score, matching the 415 time-points for which a vaginal ring was available. FISH results were available for 362 vaginal slides matching the 415 time-points for which a vaginal ring was available. The quality of 53 samples was not sufficient for FISH.

Vaginal microbiome

Most slides (61.5%) had a normal Nugent score of 0-3, 28.9% a BV Nugent score 7-10, and 9.6% an intermediate score of 4-6. Fluorescence microscopy results are shown in Table

Table 4.11: Vaginal microbiome of participants at time of removal of contraceptive ring: presence and absence of a vaginal biofilm with confocal laser scanning microscopy after fluorescence in situ hybridisation by species. (53 results unavailable due to inadequate quality of samples for confocal laser scanning microscopy)

Fluorescence microscopy characteristic N=362	n (%)
Presence of vaginal biofilm	
All bacteria	192 (53.0)
Gardnerella vaginalis	139 (38.4)
Atopobium vaginae	98 (27.1)
Presence of dispersed species only	
All bacteria	170 (47.0)
Gardnerella vaginalis	71 (19.6)
Atopobium vaginae	40 (11.0)
Absence of species	
All bacteria	0 (0)
Gardnerella vaginalis	152 (42.0)
Atopobium vaginae	224 (61.9)

4.11. A bacterial biofilm was present on 53% of vaginal slides, a *G. vaginalis* biofilm in 38.4% of samples, and an *A. vaginae* biofilm in 27.1% of slides (Table 4.11).

Presence, bacterial composition, and structure of the vaginal ring biomass

All 415 rings were evaluated for the presence of biomass using the crystal violet assay, and all of them tested positive. The biomass OD ranged from 0.13 to 3.92 (mean OD 3.35; standard deviation (SD) 0.64). qPCR showed that the *Lactobacillus* genus was present in most ring eluates (384/412; 93.2%), with a mean $\log_{10}$ bacterial concentration of 6.22 geq/ml (SD 0.98). *G. vaginalis* was detected in 237 eluates (57.4%; mean concentration 6.05 geq/ml - SD 1.17). *A. vaginae* was less common and quantified in 154 samples only (37.3%; mean load 6.69 geq/ml - SD 1.33). Ninety ring biomass eluates contained *G. vaginalis* without *A. vaginae* being present whereas *A. vaginae* was only detected in seven ring eluates without *G. vaginalis*. The images of the fluorescence microscopy, on a subset of 120 ring biomass samples mounted on slides, showed a presence of lactobacilli in 77 (64.2%) of samples. *G. vaginalis* was seen in 74 (61.7%) and *A. vaginae* in 37 (30.8%) of the biomass samples mounted onto slides (Figure 4.8).

The presence of *A. vaginae* in the ring biomass eluate was associated with the biomass density (OD +0.18; 95% CI 0.05-0.32; p=0.009) and showed a significant linear increase (OD +0.03; 95% 0.01-0.05; p=0.002) for each $\log_{10}$ increase in *A. vaginae* concentration. The presence of *G. vaginalis* was not significantly associated with the ring biomass density (OD +0.10; 95% CI -0.03-0.23; p=0.130), but the ring biomass density increased for each

$\log_{10}$ increase in *G. vaginalis* concentration (OD +0.03; 95% CI 0.01-0.05; p=0.013). For the *Lactobacillus* genus, neither the presence (OD -0.03; 95% CI -0.28-0.22; p=0.822), nor the concentration (OD +0.00; 95% CI -0.03-0.04; p=0.983) was correlated with the ring biomass density.

SEM on a subset of 11 rings showed that all rings were covered with layers of vaginal epithelial cells and bacteria with diverse shapes and sizes were seen adhering to these epithelial cells. We differentiated two phenotypes (Figure 4.9). The first type consisted of a loose network of scattered elongated bacteria. The second type was characterised by a dense bacterial biofilm with bacilli. All seven rings categorised in the first phenotype had matching vaginal samples that were scored as Nugent 0-3 (n=6) or 4-6 (n=1), while the three rings with phenotype 2 had matching vaginal samples scored as Nugent 8-10.

Association of the vaginal microbiome compositions and the ring biomass density

Mean ring biomass densities were compared among the three Gram stain Nugent score categories (for rings and Gram stain slides that were collected together: for the same participant at the same study visit). Vaginal ring biomass in the BV Nugent score category (OD +0.26; 95% CI 0.11-0.41) and intermediate score category (OD +0.09; 95% CI -0.12-0.30) had a statistically significantly higher density compared to ring biomass in the normal score category (p=0.002) (Table 4.12). The presence of a vaginal biofilm containing either *G. vaginalis* and/or *A. vaginae* by FISH fluorescence microscopy also correlated with a higher ring biomass density (OD +0.24; 95% CI 0.10-0.38; p=0.001 and OD +0.22; 95% CI 0.06-0.37; p=0.006 respectively); Table 4.12). No significant associations between the presence of planktonic/dispersed vaginal *G. vaginalis* and *A. vaginae* and ring biomass density were found.

4.5.4 Discussion

This laboratory sub-study of a vaginal contraceptive ring trial in African women showed that the formation of biomass on the vaginal rings that had been worn for three weeks was common and present in varying densities. We demonstrated that lactobacilli were nearly always part of the ring biomass and that bacteria playing an important role in BV were often present: *G. vaginalis* in more than half of the ring biomasses and *A. vaginae* in more than one-third. The concentrations of these two bacteria in ring eluates were positively associated with ring biomass density, indicating that a denser biomass likely consists of higher numbers of the bacteria. In addition, we showed that vaginal microbiota dysbiosis (defined as a Nugent score of 7-10 and 4-6) or vaginal biofilm presence (visualised by

Table 4.12: Association of the vaginal microbial status with contraceptive vaginal ring biomass.* corrected for participant multiple observations and randomisation group.

Risk factor	Mean change in density for a one unit change in risk factor	95% confidence interval	p-value from regression analysis
Diagnosis bacterial vaginosis			0.002
Normal Nugent score (0-3)	Ref	-	
Intermediate Nugent score (4-6)	+0.09	-0.12 - 0.30	
Bacterial vaginosis Nugent score (7-10)	+0.26	0.11 - 0.41	
Fluorescence microscopy after FISH by species			
Gardnerella vaginalis biofilm	+0.24	0.10 - 0.38	0.001
Gardnerella vaginalis dispersed only	+0.10	-0.04 - 0.24	0.147
Atopobium vaginae biofilm	+0.22	0.06 - 0.37	0.005
Atopobium vaginae dispersed only	+0.09	-0.05 - 0.24	0.195

fluorescence microscopy) were associated with higher ring biomass densities. These findings suggest that the status of the vaginal microbiome influences the formation or deposit of biomass on vaginal rings and/or vice versa. Our study was cross-sectional and therefore does not allow us to determine temporality and causality of these associations.

Only two other human studies and one macaque study have visualised the surfaces of vaginal rings after use. Miller et al. applied electron microscopy to examine a NuvaRing used for four weeks by a healthy volunteer, and observed cellular debris but no bacterial growth on the surface of the ring [409]. We speculated that the magnification of 200X that they used was too low to visualise bacteria. In comparison, we used magnifications of 1000X and 4000X in the present study. A second study in human volunteers showed the presence of biomass on all 48 rings containing an antiretroviral drug that were used for four weeks [411]. SEM with a magnification of 25X was used to semi-quantify the biomass density. In this population of women, of whom more than two-third had a normal Nugent score, the ring biomass density (semi-quantified visually with SEM) was not associated with the Nugent score category [411]. Gunawardana et al. [221] differentiated two biomass phenotypes, while visualising the surface of vaginal rings worn by six female pig-tailed macaques for 28 days with electron microscopy and fluorescence microscopy. They found large areas of the ring surface covered with tightly packed mats of bacteria and epithelial cells or thicker interwoven networks of uniform fibres. We also differentiated two phenotypes. The first type consisted of a loose network of scattered elongated bacteria, probably lactobacilli, which agrees with the normal Nugent score of the matching vaginal smears. The second type was characterised by a dense bacterial biofilm with bacilli, also in agreement with the BV Nugent score of the associated vaginal smear.

At present, contraceptive vaginal rings are commonly used in countries where HIV is

not endemic and BV prevalence in low. However, multipurpose and long-acting vaginal rings for the prevention of HIV and pregnancy are being developed specifically for in HIV-endemic countries, most of which are in sub-Saharan Africa [67, 72, 440, 446–448]. Recently, a vaginal ring containing the antiretroviral drug dapivirine was shown to be effective for HIV prevention in sub-Saharan African women and this monthly ring will soon be licensed for that purpose. It will be the first vaginal HIV prevention option for women at risk of HIV infection [439]. However, BV prevalence in sub-Saharan Africa is high, and biomass deposit on these HIV prevention rings might promote or maintain vaginal dysbiosis in ring users and/or hamper active drug release from the rings, resulting in reduced efficacy [2]. This requires further study. Extensive epidemiological research has shown that sex hormones, including those released by contraceptive vaginal rings, have a beneficial effect on the vaginal microbiome [69, 70, 72]. Our data confirm this: we observed an improvement in the vaginal microbiome by Nugent score after initiation of NuvaRing use (data not presented). Incorporating oestrogen and/or progestogens in vaginal rings may therefore be an important strategy to protect the vaginal microbiome during ring use in addition to protecting against pregnancy. Other components that are beneficial for the vaginal microbiome, such as acidifying agents and probiotic lactobacilli, could also be added to vaginal rings in the future.

Several types of silicones and thermoplastics are used to manufacture vaginal devices. We have previously shown that the ability of bacteria to adhere to the device surfaces differs by the type of material used [449]. We studied *Neisseria gonorrhoeae* biofilm on silicone and thermoplastic vaginal ring materials and showed the adherence of gonococci was greater on the silicone ring material as compared to the thermoplastic ring material [449]. The NuvaRing is composed of a thermoplastic (ethylene-vinyl acetate copolymer) material, which was less prone to colonisation by gonococci. However, it is unclear if BV-associated bacteria would behave similarly to gonococci in vitro, and if the in vitro data accurately predict what would happen in vivo.

In summary, our study showed that biomass easily forms on the contraceptive vaginal ring within three weeks and that BV-associated bacteria are commonly present in this biomass. Our study also showed associations between the presence of vaginal dysbiosis and vaginal biofilm and the the ring biomass density. The temporality and causality of these relationships deserve further study. Furthermore, we recommend that the design and development of multipurpose vaginal rings take ring biomass formation into account by studying the effects on the vaginal microbiota and active product release.

Figure 4.8: Visualisation of biomass on intravaginal ring surface by confocal laser scanning microscopy after fluorescence in situ hybridisation at 400x magnification: A. Lactobacilli scattered on vaginal epithelial cells; B. Vaginal epithelial cells covered with bacterial biofilm.

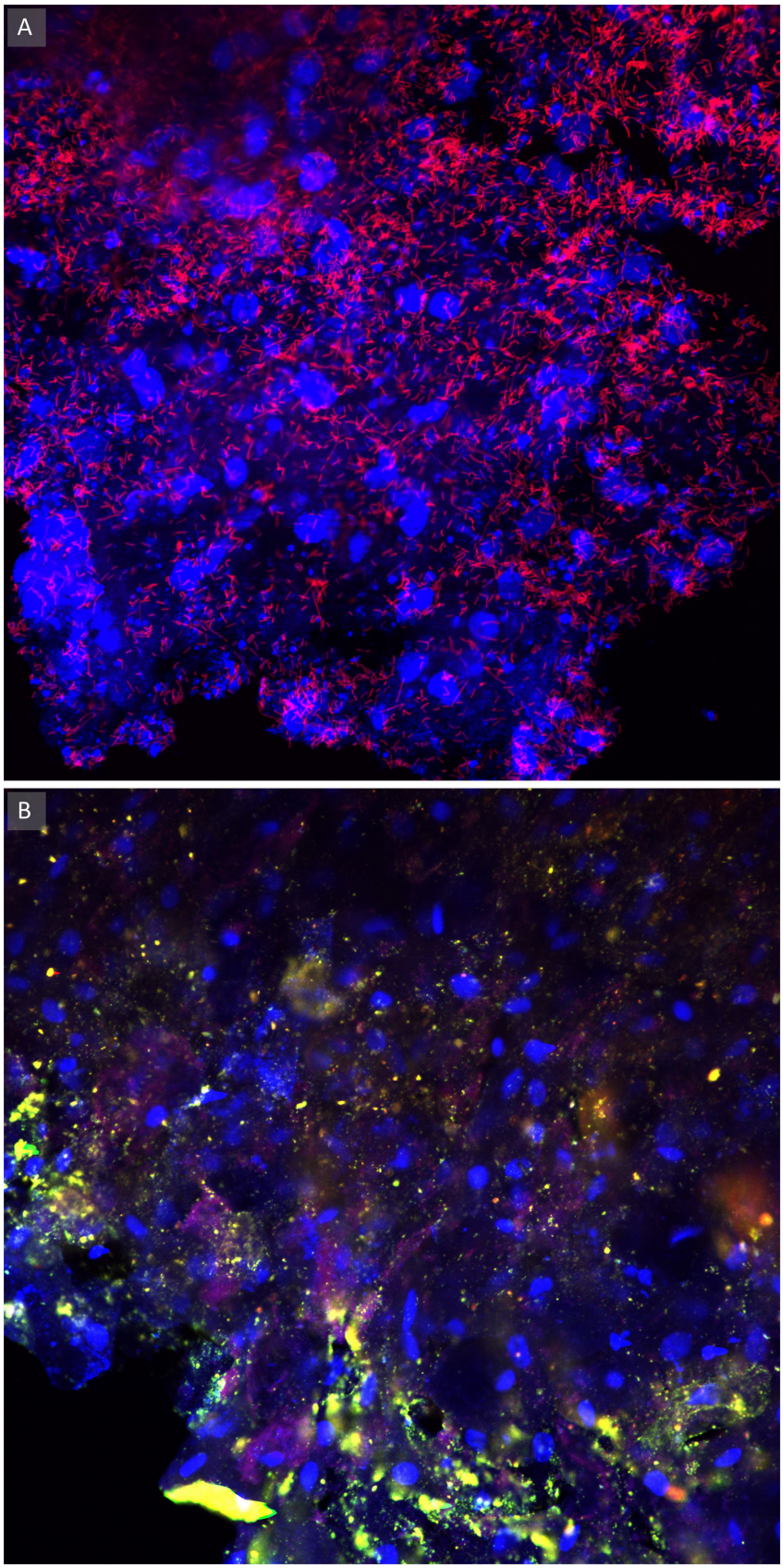
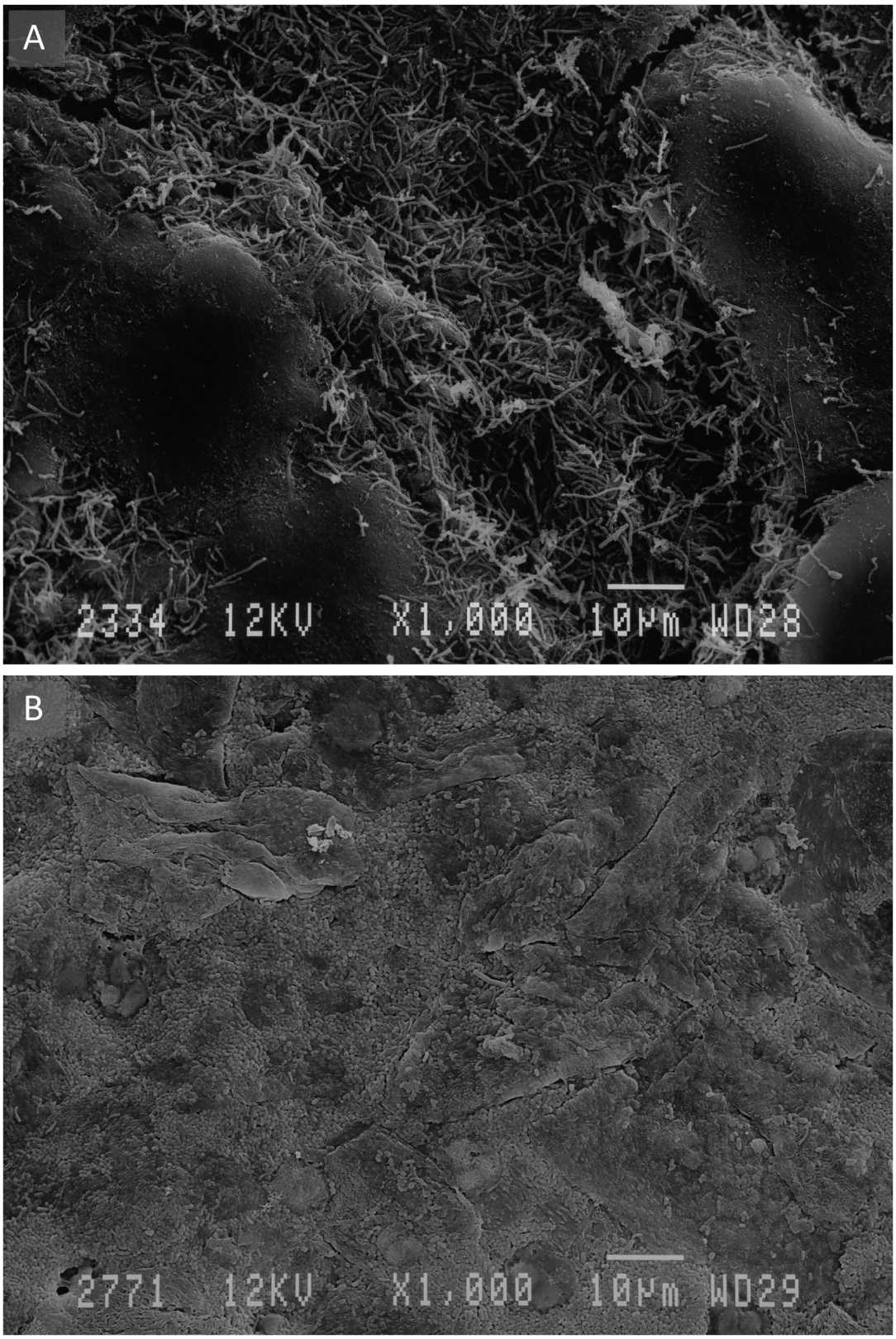


Figure 4.9: Visualisation of biomass on intravaginal ring surface by scanning electron microscopy at 1000x magnification: A. Phenotype 1 - elongated bacteria scattered on vaginal epithelial cells; B. Phenotype 2 - condense biofilm of bacilli on vaginal epithelial cells.



CHAPTER 5

General discussion

There are two major vaginal microbiome states: the healthy vaginal microbiome which is dominated by lactobacilli, and the bacterial vaginosis (BV) vaginal microbiome which is described as polybacterial dysbiosis. The lactobacilli (with the exception of *Lactobacillus iners*) are considered to disappear in BV, while the bacterial load of other (facultative) anaerobic bacteria, such as *Gardnerella vaginalis* and *Atopobium vaginae*, increases [15]. It also has been demonstrated that *G. vaginalis* and *A. vaginae* are important constituents of a biofilm on the vaginal epithelium, that is associated with BV [9, 141, 209, 444]. Moreover, *L. iners* has been shown to increase during menstruation [61], during BV [47] and with sexual exposure [42], but the ambiguous role of this lactobacillus is not the focus of this research.

In chapter 4.2 and 4.3 we described how we designed and applied a new specific PNA probe for *A. vaginae*, AtoITM1, on a range of vaginal slides together with an already existing *G. vaginalis* and a broad-range bacterial PNA probe to investigate the role of *G. vaginalis* and *A. vaginae* in vaginal biofilm. The gathered data on biofilm were compared with the quantitative real-time polymerase chain reaction (qPCR) and Nugent score data to look for associations between the presence of vaginal biofilm and dysbiosis. Furthermore, we attempted to differentiate between two types of *G. vaginalis*: the harmless one, residing in the healthy vaginal microbiome, and the biofilm-forming one, associated with vaginal dysbiosis. We looked into the association of *G. vaginalis* with the anaerobic bacterium *A. vaginae* (chapter 4.3). In chapter 4.4 we investigated the role of *G. vaginalis'* ability to produce sialidase in the development of a biofilm on the vaginal epithelium. Finally, we

applied the developed and optimised techniques to investigate the influence of the vaginal status on the colonisation of intravaginal contraceptive rings. In chapter 4.5 we explored the adherence of vaginal epithelium and associated microbes to these intravaginal rings and defined risk factors for a denser ring biomass.

We have succeeded in laying bare more of the biofilm's characteristics and we have highlighted the importance of *G. vaginalis* and *A. vaginae*, using fluorescence in situ hybridisation (FISH). Furthermore, we have demonstrated that the presence of the *G. vaginalis* sialidase gene is linked with BV and *G. vaginalis* biofilm and that this vaginal state is associated with the deposit biomass on vaginal contraceptive rings.

Our study also has several limitations. We used samples of a small group of 120 women who were followed up for a maximum of 13 weeks. No control or placebo group was available, which implicates that all participants used a contraceptive ring for the bigger part of the study. However, we did have a baseline sample for each of the participants as a reference for their vaginal microbiome without the influence of contraceptive ring. Our newly designed FISH probe targeting *A. vaginae* has a rather low sensitivity, which might result in an underestimation of the presence of *A. vaginae* in the biofilm-positive samples. Furthermore, microscopic evaluation after FISH is (like Nugent scoring) a subjective technique and has some technical difficulties (for example background signal), which we tried to handle by using evaluation criteria and assessing multiple fields. Due to the study setup, we did not have access to bacterial cultures which could be used in the assessment of sialidase production by *G. vaginalis*.

Finally, we used a range of molecular and microscopic techniques to explore the vaginal biofilm and the ring biomass, but we could have gained even more information using other technologies that would enable us to broaden our spectrum. Notwithstanding this narrow approach, we gained valuable information concerning two main players in BV, while acknowledging that they are only a small piece of the puzzle.

5.1 Visualising vaginal biofilm with fluorescence in situ hybridisation

The best-known application of FISH is in cytogenetics: the detection of specific DNA sequences on chromosomes for the prenatal diagnosis of developmental diseases or for identification of cancer cells. It is also widely used in microbial ecology to identify specific microorganisms (for example in complex biofilms).

In our studies, we have used peptide nucleic acid (PNA) probes instead of the DNA alternative: PNA has a neutral backbone giving it a significant advantage in low ionic-strength

conditions, that could hinder the complementary genomic sequences from reannealing while performing FISH [208]. Additionally, the PNA probes are superior in penetrating the cell wall and hydrophobic cytoplasmic membrane of the target organism [207]. A wide range of fluorochromes is available, and we selected Alexa fluorochromes, which have spectral properties similar to other fluorochromes such as cyanine dyes, but are brighter and more resistant to photo bleaching [421].

5.1.1 Existing probes for FISH in BV research

Already more than 10 years ago, in 2005, Swidsinski et al. [9] published the first work employing FISH in BV research. They applied FISH on vaginal biopsies using up to 35 different DNA probes targeting mainly bacteria residing in the gastrointestinal tract. Out of these 35, only three specific probes were positive in the majority of the samples: the probes targeting *Lactobacillus* spp. (Lab158), *G. vaginalis* (GardV, derived from Bif662), and the group of *Atopobium, Coriobacterium, Eggerthella* and *Collinsella* spp. (Ato291) [9]. In the same year, Fredricks et al. [167] demonstrated the use of FISH using the same probe, Ato291, for *A. vaginae* and a newly designed one for *G. vaginalis* (G.vag-198). They also designed probes for other BV-associated bacteria: the thus far unknown BVAB 1 (Uncxb1-132), BVAB 2 (Uncxb2-1244), and BVAB 3 (Uncxb3-1244), and *Mobiluncus* (Mobil-126) [167]. Later, Swidsinski et al. added two probes against *Bacteroides/Prevotella* spp. (Bac303) and the Enterobacteriaceae cluster (Ebac21) to the panel [450], modified the probe targeting *G. vaginalis* [305] and refined their method on urine samples [305].

The first PNA probes were designed by Machado et al., to detect *Lactobacillus* spp. (Lac663) [451] and *G. vaginalis* (Gard162) [204]. Both probes have been used extensively in vivo [205] and in vitro [261, 265] for BV research.

Using the Gard162 PNA probe, we obtained clear hybridisation for all *G. vaginalis* isolates tested and observed no cross-reaction with strains of the other species. Used on clinical samples and compared with the qPCR results, the *G. vaginalis* probe had a sensitivity of 86% and specificity 75%, which was lower than reported by Machado et al. [204].

For detection of *A. vaginae*, Swidsinski et al. [9] employed a DNA probe (Ato291), designed by Harmsen et al. [415] for the detection of species of the *Atopobium* cluster in faecal samples and based on the sequences of Coriobacteriaceae strains isolated from faeces and clinical material. We used an Ato291 equivalent PNA probe, but it showed low specificity on vaginal clinical isolates. Consequently, we designed and tested a new probe for *A. vaginae* targeting the 16S rRNA-gene, based on published PCR primers [195]. This AtoITM1 probe showed an excellent specificity of 90% but a limited sensitivity of 67%. The relatively low sensitivity of the *A. vaginae* FISH assay cannot be explained by

the bacterial load as measured by qPCR. One possible explanation could be the typical structure of a biofilm, where an oxygen gradient exists from the surface to the centre of the biofilm [423]. This kind of oxygen gradient can develop in bacterial biofilms, due to the consumption of oxygen by bacteria at the surface of the aggregate. It offers an ideal opportunity for strict anaerobes, like *A. vaginae* to persist in the centre of the aggregate, away from the oxygen source [338]. In the vaginal environment, oxygen diffuses both from the vaginal smooth muscle into the lumen of the vagina and from the lumen to the basal side. This possibly results in *A. vaginae* being closely embedded by *G. vaginalis* in the centre of the biofilm and not appearing at the surface or close to the vaginal epithelial cells. It could be that the PNA probes are not able to fully penetrate into the inner parts of the biofilm. Another explanation might be that the PNA probes do penetrate, but that the fluorescence could be masked and not be detected due to the microscope's low resolution. Moreover, both explanations are not mutually exclusive.

Vaginal slides, processed directly after sampling, proved to be a valid sample type for imaging the composition of the vaginal microbiome, as shown earlier by Peltroche-Llacsahuanga et al. [422]. Vaginal swabs are easy to collect, and slides can be stored at room temperature for at least six months after heat fixation (based on our experience), which makes it an ideal sample type for BV research. In our opinion, vaginal slides make a better type of sample to use in BV research than urine samples, that are being used by Swidsinski et al. [9]. When we compared both types of samples, the majority of the urine samples did not contain vaginal epithelial cells, which made a proper analysis of these samples impossible. We did not encounter this problem with the same magnitude when using vaginal smears, although the quality of the smears (not too thick!) was also an important success factor for FISH.

FISH proved to be a useful method to identify and localise bacteria, that could easily be embedded in BV research projects. Despite the value of FISH in research, it requires a well-equipped laboratory and a skilled and experienced microscopist to interpret the results. Therefore, translating the technique to the clinical practice, in regard to providing a more accurate method for diagnosis of BV, is not something for the foreseeable future.

5.2 *G. vaginalis*: mostly harmless?

The presence of *G. vaginalis* in the vaginal microbiome of healthy women is contradictory to its role in BV. In 1955 already, Gardner and Dukes [245] pursued to confirm Koch's

postulates[1] by transferring *G. vaginalis* into the vagina of a women with a *Lactobacillus* dominated vaginal microbiome, but the subject did not develop BV. However, when a BV patient's vaginal fluid was transferred into a healthy vagina, BV developed in this healthy volunteer [245]. One explanation might be that the vaginal fluid of the BV patient contained biofilm-infested vaginal epithelial cells, that could contain more virulent strains of *G. vaginalis* than the laboratory strain used in the first experiment. The existence of different types of *G. vaginalis*, more and less virulent ones, has been a popular subject of study in the BV field.

G. vaginalis can inhabit the genital tract of healthy women [3, 142, 167, 433, 452], but there seems to be a big difference in concentration when compared to the BV vaginal microbiome. The numbers of *G. vaginalis* in the healthy vaginal microbiome are several logs lower than the numbers found in the BV vaginal microbiome [19, 61, 171, 452]. Moreover, certain strains are more likely to be associated with BV, and these strains are significantly more cytotoxic than non-BV isolates [258].

The involvement of *G. vaginalis* in the vaginal epithelial biofilm has been recognised by Swidsinski et al. [9]. They also showed that only biofilm-forming *G. vaginalis* isolates were present in partners of women with BV. This could signify that not the presence of *G. vaginalis* in the vagina, but merely the occupancy by biofilm-forming *G. vaginalis* isolates is associated with BV and that these strains can be sexually transmitted [305]. Harwich et al. [258] showed that the BV isolates were more able to adhere to cultured cervical epithelial cells. This was confirmed by Castro and colleagues [453], who demonstrated that BV isolates were able to adhere in high densities to a HeLa cell line [2]. In another study by Patterson et al. [260], adherence to vaginal epithelial cells by *G. vaginalis* was higher compared to the other BV-associated anaerobes, but no comparison with isolates from the healthy vaginal microbiome was made.

Results from genomic sequence analysis could also identify significant differences between strains isolated from the healthy and BV vaginal microbiome. One difference was seen in the vly gene (encoding vaginolysin) and another in a gene encoding a cell wall-anchored adhesin, which is a biofilm-associated protein (BAP) [258]. Another comparative genomic study revealed that BV isolates encoded a large number of proteins, including enzymes enabling mucin degradation, that were not found in isolates from the healthy vaginal microbiome [429]. The BV isolates also possessed a broader group of antibiotic resistance genes, including an aminoglycoside phosphotransferase and the precursors of methicillin

[1]Koch's postulates: four criteria to identify the causative agent of a particular disease: 1) the microorganism must be present in all cases of the disease; 2) the pathogen can be isolated from the diseased host and grown in pure culture; 3) the pathogen from the pure culture must cause the disease when inoculated into a healthy, susceptible subject; 4) the pathogen must be reisolated from the new host and shown to be the same as the originally inoculated pathogen.

[2]HeLa cell line: an immortalised cell line derived from cervical cancer cells of Henrietta Lacks

resistance [429].

So, genotypic and phenotypic diversity within *G. vaginalis* has been described in terms of virulence factors, in particular the production of biofilm, as well as in the production of sialidase [160].

5.3 Production of sialidase by *G. vaginalis*

One of *G. vaginalis'* virulence factors is its ability to produce and use sialidase (neuraminidase). Sialidase facilitates the destruction of the protective mucus layer in the vagina by hydrolysis of sialic acid, which is frequently the most distant monosaccharide moiety of the glycoconjugates of mucous epithelial membrane cells and of mucines [293,295,454]. After being exposed by sialidase, these glycoconjugates can serve as receptors for the bacterial cells which promotes the adhesion phase of biofilm development [295]. Furthermore, sialidase helps to circumvent the vaginal innate and adaptive immune response, since it modulates the activity of sialylated immune mediators such as the complement factors, interleukins, immunoglobulins and various cellular receptors [297].

Only certain genotypes of *G. vaginalis* can produce sialidase [160]. We designed and validated new qPCR primers targeting the sialidase gene of *G. vaginalis* and linked the qPCR outcomes with the Nugent score and the FISH results for these samples. The *G. vaginalis* sialidase gene was present in about 75% of the *G. vaginalis*-positive samples, and in about 60% of those samples a high load ($>10^6$ geq/ml) of the gene was detected. This prevalence is high compared to results from Lopes and colleagues [160] who detected the *G. vaginalis* sialidase gene in 51% of the *G. vaginalis* strains isolated from Belgian women. However, it may be explained by the fact that the prevalence of vaginal dysbiosis in our study population is probably higher than in the Belgian group of women (although BV prevalence was unknown for this last group).

We demonstrated that the presence of a high concentration of the *G. vaginalis* sialidase gene was significantly associated with the diagnosis of BV according to the Nugent score and with the presence of a vaginal epithelium biofilm as detected by FISH. Consequently we hypothesised that one of the factors that differentiates between the type of *G. vaginalis* found in the asymptomatic vaginal microbiome and the BV-associated *G. vaginalis* is the presence of the sialidase gene and *G. vaginalis'* ability to produce sialidase. This hypothesis will need to be verified in a larger study, more specifically investigating *G. vaginalis* in both types of vaginal microbiome and linking its presence with vaginal complaints. Furthermore, we suggested that *G. vaginalis* uses sialidase to initiate adherence to the vaginal epithelium, which also warrants deeper investigation.

5.4 With a little help from my friends: *A. vaginae*

Mixed-species biofilms are the dominant form in nature and are prominent in the human body [7, 338]. Bacteria in a polymicrobial biofilm interact with their neighbours, thereby creating more beneficial living conditions for the members of the biofilm.

G. vaginalis is currently assumed to be one of the dominant members of the bacterial biofilm in BV. Even though it has been shown to have higher virulence potential than any of the other tested BV-associated bacteria [260], it is currently still unclear whether *G. vaginalis* could be capable of causing BV on its own. Machado et al. [261] recently demonstrated that *G. vaginalis* derived a growth benefit from the addition of a second species to an in vitro adhesion assay. Regardless of which second bacterial species was added, growth and adhesion of *G. vaginalis* was increased. This is in agreement with an earlier study of Pybus and Onderdonk [455], who revealed a symbiotic relationship between *G. vaginalis* and *P. bivia*, which could contribute to the progression of BV. *P. bivia* produces ammonia during its growth, which is then utilised by *G. vaginalis*. On the other hand, during *G. vaginalis* growth, amino acids are produced that can be used by other anaerobes, such as *P. bivia* [455, 456]. Machado et al. [261] also showed a symbiosis between *G. vaginalis* and *Fusobacterium nucleatum*. Although a limited amount of research has been done on the role of *F. nucleatum* in BV, it has been shown to play a key role as bridging species in the establishment of oral biofilms. Foster and Kolenbrander [457] demonstrated that in vitro *F. nucleatum* is not capable of developing a biofilm on its own, but *F. nucleatum* can become a dominant member of oral multispecies biofilms by co-aggregating with pathogenic bacteria. In the study of Machado et al. [261], *F. nucleatum* was also able to join the initial biofilm formed by *G. vaginalis* and establish a symbiotic relationship with *G. vaginalis*. Synergistic cooperations between *G. vaginalis* and *M. hominis* [458], and *G. vaginalis* and *A. vaginae* [444] have also been demonstrated using clinical samples.

In chapter 4.3, we confirmed the findings of earlier studies [9,209] concerning the importance of both *G. vaginalis* and *A. vaginae* in the vaginal epithelial biofilm. Furthermore, we demonstrated the significance of *A. vaginae* and the synergy between *A. vaginae* and *G. vaginalis* in vaginal dysbiosis, using highly specific PNA probes for both species. Additionally, in chapter 4.2 we showed that higher bacterial loads of *G. vaginalis* and *A. vaginae*, as detected by qPCR, are associated with a higher probability of the presence of a bacterial biofilm.

We also showed in chapter 4.3 that *A. vaginae* was almost always accompanied by *G. vaginalis* in the BV biofilm: we only found two samples containing *A. vaginae* in the absence of *G. vaginalis*, while more than one-third of the *G. vaginalis*-positive samples was negative for *A. vaginae*. This is in accordance with prior reports on the association of *A.*

vaginae with *G. vaginalis* [9,164,165,171,209]. Moreover, the odds ratio of having a Nugent score higher than 3 (intermediate vaginal microbiome or BV) was highest when both *A. vaginae* and *G. vaginalis* were part of a biofilm on the vaginal epithelium. However, the presence of both bacteria, regardless of their existence in a biofilm, was also associated with BV according to Nugent. We also showed in chapter 4.2 that the mere presence of *A. vaginae* did not simply predispose to the presence of a polymicrobial biofilm, but when *A. vaginae* was part of the biofilm, compared to a biofilm of only *G. vaginalis*, both bacterial species were present in higher concentrations.

The involvement of *A. vaginae* in BV has only recently been established [163, 165, 166] and we have now confirmed the findings of Swidsinski et al. [9] that *A. vaginae* is part of the BV biofilm. *A. vaginae* can induce an inflammatory response [334], but it did not demonstrate any specific virulence factors in vitro [261]. The presence of *A. vaginae* could have a major impact on treatment since susceptibility to metronidazole, the standard treatment for BV, varied significantly across various *A. vaginae* strains in vitro [330]. In vivo data are still scarce, but Bradshaw et al. [170] found that BV recurrence rates were higher when *A. vaginae* was present in the vaginal microbiome in addition to *G. vaginalis*. Another study by Ferris et al. [163] demonstrated that a high concentration of *A. vaginae* before treatment was associated with complete or partial failure of treatment for BV. In addition, bacteria in a biofilm are less sensitive to antibiotic treatment [345]. On top of the observation that some *A. vaginae* isolates are metronidazole resistant, the association of this species with BV biofilm can explain the problems with treatment of BV and the recurrent nature of the condition.

These findings might be of importance for the diagnosis of BV. It has already been established that the sole presence of *G. vaginalis* is not a good enough marker to be used for the detection and diagnosis of BV [40, 41, 158]. However, the presence of both *G. vaginalis* and *A. vaginae* was demonstrated to have a higher predictive value for the diagnosis of BV [171,174,459]. One might even argue not to use the presence of *G. vaginalis* as a marker of BV, but instead target diagnostic methods against the sialidase gene of *G. vaginalis*, since the presence of this gene correlated with the detection of a vaginal biofilm. Multiplex tests have been proposed and tested by different research groups [197–199]. Currently, the detection of specific bacterial species of specific genes is mostly being done by molecular methods, which still need well-equipped laboratory and trained analysts, making this not really useful for the clinical practice. However, we are only a small step away from the development of easy-to-use point-of-care tests, since the knowledge and the technology are available.

5.5 Treatment of BV

There is still a sustainable amount of information about the BV-associated biofilm to be uncovered, but even without these details about the involved bacteria and mechanisms, we are already aware of the impact the biofilm has on treatment of BV. The available antibiotics do not succeed in curing the condition, as they are still mainly directed toward alleviation of symptoms [460], and very high BV recurrence rates have been reported [461]. Moreover, the existence and impact of a polymicrobial vaginal biofilm is not taken into consideration when treating BV at this moment. However, candidates for biofilm disruptors have already been included in more recent research into anti-BV agents [461].

A possible approach to deal with BV is the restoration of the vaginal environment by the administration of live microorganisms, or probiotics. The administration of *Lactobacillus* strains could facilitate the inhibition of bacterial adhesion to the vaginal epithelium [204] and the production of antimicrobial compounds, such as lactic acid [32,33] and bacteriocines [36] can have a bactericidal effect on the BV-associated anaerobes. Formulations containing probiotic lactobacilli strains are currently being investigated as a stand-alone therapy or as adjuvants to antibiotic therapy [238, 461]. In vitro studies have demonstrated that probiotics could be effective against the BV biofilm. Saunders et al. [462] have shown that *L. reuteri* RC-14 could disrupt *G. vaginalis* biofilms. This was confirmed by McMillan and colleagues [463], who in addition also studied the disruptive effects of *L. rhamnosus* on the biofilm in vitro.

To enhance the effect of probiotics, they can be administered in combination with prebiotics[3]. Natural antimicrobials, mainly bacteriocines, could also have a therapeutic effect against BV [461]. Turovskiy et al. [464] showed that out of a wide range of bacteriocines, lauramide arginine ethyl ester (LAE) had the strongest bactericidal effect against *G. vaginalis* biofilms, while not affecting the lactobacilli. According to Algburi in vivo [465], LAE could enhance the action of antibiotics clindamycin and metronidazole, when used as a combination therapy.

A novel approach, DNase, targets the extracellular DNA in the biofilm that ensures its structural integrity. Moreover, DNase would destroy the biofilm and liberate bacteria, which could subsequently be killed by antibiotics, when used in combination with DNase [232]. Some other strategies to destroy the biofilm and treat BV, could be acidifying the vaginal environment [33], synthetic antimicrobial peptides [466], the application of antiseptics [467] and plant-derived compounds [468], and the destruction of the biofilm matrix [469]. Combination therapies, that combine the disruption of the biofilm matrix with specific bactericidal effects, or a bactericidal antibiotic combined with the restoration of the vaginal

[3]Prebiotics: nondigestible carbohydrates that act as nutrition for probiotics

lacobacilli, will likely be most effective.

An approach that is still understudied is the use of bacteriophages [4] in the treatment of BV, although a few studies in other biofilm-associated infections have already been carried out [470]. Phage therapy could provide a natural, highly specific and safe approach for controlling BV-associated bacteria, if the phages are able to reach the biofilm in sufficient numbers [471,472]. Controlled infection with a mixture of bacteriophages would result in the killing and lysis of specific targeted bacteria. This process of active penetration of phages in the biofilm has an impact on the structure of biofilms and promotes the release of new phage virions, that will continue to infect adjacent bacteria [471,472]. Additionally, certain bacteriophages can express extracellular polysaccharide (EPS) depolymerase enzymes, either naturally [473,474] or as a result of bioengineering [475], that could also contribute to the degradation of the biofilm structure. However, currently no bacteriophages for the BV-associated bacteria have been described, and the interactions of natural bacteriophages with the extracellular matrix of the BV biofilm will also need to be studied more extensively before this approach could be taken into consideration.

And finally, another understudied approach to eradicate the BV biofilm would be the interaction with quorum sensing, or cell-cell communication. The potential of small chemical compounds to interfere with the communication between bacterial cells is being investigated, for example in *Pseudomonas aeruginosa* for the treatment of cystic fibrosis patients [400,470]. However, there is still little to no knowledge on quorum sensing in BV biofilm, let alone on how to interfere with it.

5.6 The impact of the vaginal biofilm on intravaginal contraceptive rings

Bacteria have been well documented to colonise surfaces and to develop biofilms on inert material, especially on indwelling medical devices such as catheters or prostheses inside the human body. We demonstrated that vaginal bacteria, and more specifically the bacteria involved in BV, can aggregate in a biofilm on vaginal epithelial cells, which may cause them to become less sensitive to antimicrobial therapy and allows them to persist in the vagina.

At present, intravaginal rings are available and are used worldwide for contraception, for treatment of vaginal atrophy [476] and are proposed for prevention and treatment of sexually transmitted infections, including HIV. A monthly dapivirine vaginal ring was found safe and effective in HIV prevention in sub-Saharan women and may become an

[4] Bacteriophage: virus that infects bacteria.

important HIV prevention option for women at risk of HIV infection [439]. Yet not much is known about the effects of these intravaginal rings on the vaginal microbiome or about the effects of the microbiome on the intravaginal rings.

In chapter 4.5 we presented the results of our study on the association between the vaginal microbial status and the biomass deposit on the contraceptive vaginal rings (CVRs). The density of the biomass on the vaginal rings was measured using the crystal violet assay and the biomass was visualised with fluorescence microscopy after FISH and with SEM. We showed that biomass deposit on CVRs is common and that the biomass consists of vaginal epithelial cells and members of the vaginal microbiome. In conclusion, the CVR appears to be fully "integrated" in the vaginal microbiome and becomes a part of the vaginal epithelium. We also demonstrated that the state of the vaginal microbiome has an important influence on the density of the CVR biomass: the biomass becomes denser when the Nugent score increases. This was also seen when a vaginal biofilm consisting of *G. vaginalis* and/or *A. vaginae* was visualised after FISH and when high concentrations of *A. vaginae* in the vagina were measured with qPCR. In addition, the presence of *A. vaginae* and high logs of *G. vaginalis* or *A. vaginae* on the CVR were associated with a high density of the CVR biomass. The presence of *Lactobacillus* species on the CVR did not have an effect on the CVR biomass.

Consequently, the BV vaginal microbiome has a profound effect on the development of biomass on intravaginal rings. When further developing these rings, it should be established whether the biomass on a vaginal ring can hamper the release of active products from the rings or whether the ring biomass has a negative influence on the vaginal microbiome. If future research finds that the biomass deposit on the intravaginal rings poses a threat for the safety or efficiency of the rings, it might be feasible to improve the design of the rings, for example by adding an anti-biofilm coating (if available by then). We also know that the kind of surface material can be an important factor for the growth of biofilm. In a previous in vitro experiment by our group [449], biofilm development by *Neisseria gonorrhoeae* isolates on different types of silicone and thermoplastic vaginal rings was studied. We showed a difference in adherence of bacterial cells on the two types of rings: biofilm formation was greater on the silicone rings, compared to the thermoplastic rings. This will need to be taken into account for further development and application of intravaginal rings development.

Our understanding and knowledge about the hormonal regulation of the vaginal microbiome, and more specifically in the context of individual species such as *G. vaginalis* and *A. vaginae* is limited. This thesis did not set out to study this relationship but the interactions will have implications for the use of IVR and can help in understanding the aetiology and epidemiology of BV, and treatment of the condition. It is generally accepted knowledge that hormonal contraception (combined or progesterone only) users have a significantly

reduced risk of BV defined by non-molecular methods [65, 66]. Less data is available on the association of hormones and individual species. The review by van de Wijgert [65] suggests that high levels of estradiol (due to the phase in the menstrual cycle or availability in serum of IVF patients) promote lactobacilli, and particularly *L. crispatus* [18, 60, 70, 71] which implies a lower presence of BV-associated bacteria. Similarly, pregnancy, a status of of high estradiol levels, is associated with high levels of lactobacilli, particularly *L. crispatus*, and low bacterial diversity [56, 69]. We recently presented data at the ECCMID conference defining dysbiosis by applying a score of the sum of *Lactobacillus* genus minus the sum of *G. vaginalis* and *A. vaginae* logs [477]. We showed that the score increased with evolving pregnancy; *G. vaginalis* and *A. vaginae* presence and concentrations diminished whereas lactobacilli presence and counts increased.

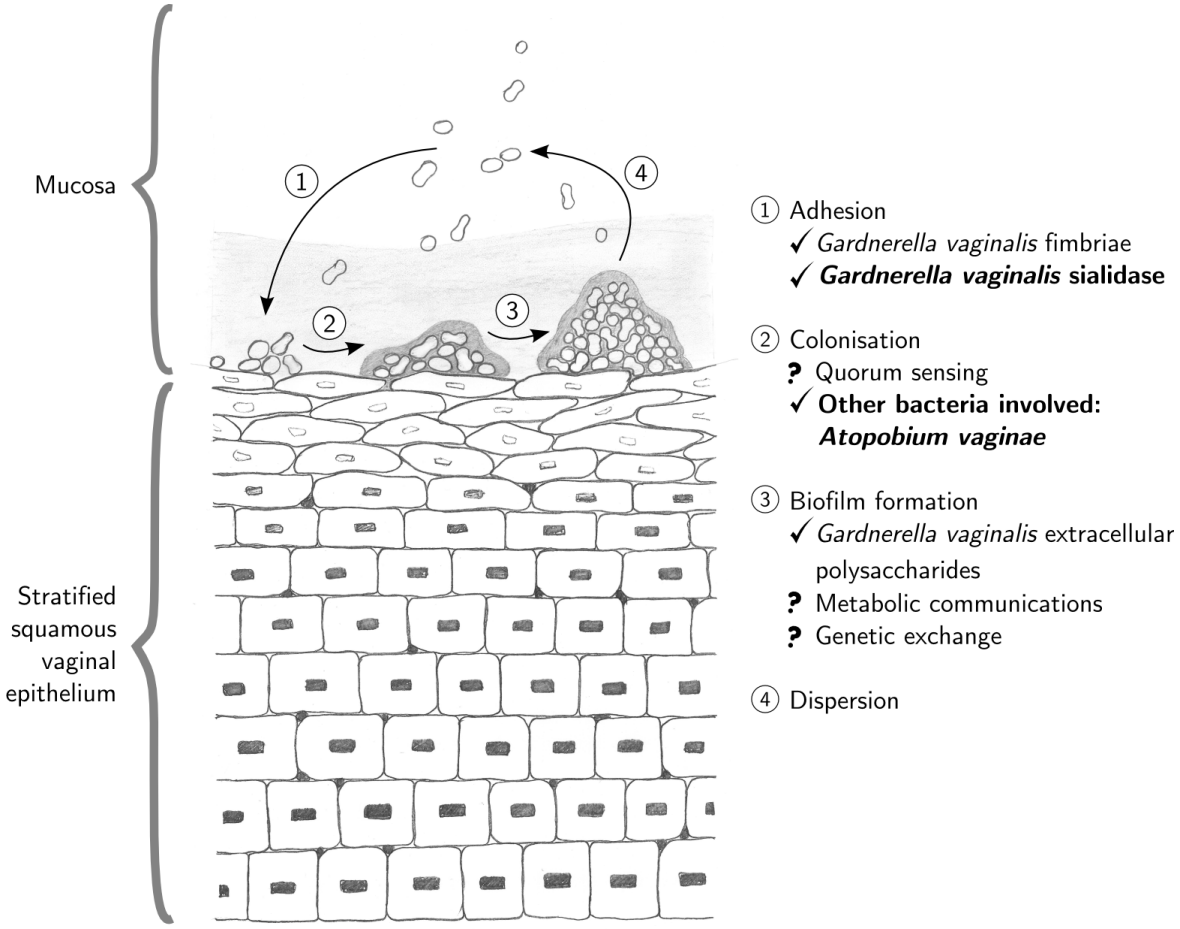
A preliminary analysis of the longitudinal data of the Ring Plus study, which is yet to be published, demonstrated a protective effect of the contraceptive ring. Over time, independently of randomisation group, the Nugent score decreased and at the end of the study there was a higher presence of lactobacilli while *G. vaginalis* and *A. vaginae* were less prevalent compared to the baseline visit. This does not necessarily contradicts the findings on the association of the dysbiosis biofilm with a higher density of the ring biomass, but highlights the positive effect of hormones on the vaginal microbiome [65, 66]. Exogenous oestrogen delivered locally in the vagina possibly has a stimulating effect on the lactobacilli population, by increasing the glycogen content of vaginal epithelial cells [66]. Furthermore, hormonal contraception reduces the amount of menstrual blood, and therefore the volume of haemoglobin that serves as an iron source for BV-associated bacteria such as *G. vaginalis* and BVAB [66, 157].

5.7 Final conclusions: the characterisation of biofilm associated with BV

In this thesis, we have described the design and optimisation of new probes, primers, and protocols for FISH and qPCR and the use of light microscopy and CLSM to characterise the biofilm associated with BV (Figure 5.1). In our attempt to discover why *G. vaginalis* can both be a member of the healthy vaginal microbiome and an important player in BV, we investigated two of *G. vaginalis'* virulence factors: biofilm development and presence of the sialidase gene.

First, we used FISH to visualise the vaginal biofilm that is associated with BV and confirmed that *G. vaginalis* is a major constituent of this biofilm and that *A. vaginae* is often involved as well. We demonstrated that high bacterial loads of these two bacteria in the vagina were associated with a higher probability of bacterial biofilm on the vaginal

Figure 5.1: Biofilm in bacterial vaginosis: what we know now



epithelium. Moreover, a polymicrobial biofilm of both *G. vaginalis* and *A. vaginae* was associated with having BV according to Nugent.

Second, we investigated the presence of *G. vaginalis'* sialidase gene and its importance in the development of a biofilm. By doing this, we could determine that the presence of *G. vaginalis'* sialidase gene was linked with the diagnosis of BV according to Nugent and with the existence of a vaginal biofilm. Sialidase is therefore strongly associated with biofilm production.

Finally, we applied our developed techniques and acquired knowledge to study the effect of these vaginal biofilms on intravaginal rings. Growth on the CVRs was common and this growth reached a higher density when a vaginal biofilm was present or high concentrations of *G. vaginalis* or *A. vaginae* were detected on the CVR. Therefore, we concluded that the BV vaginal microbiome increased the risk of biomass development on inserted medical devices, which may be important in terms of safety and efficacy of such devices when used on a large scale.

5.8 Directions for future research

We have demonstrated that the presence of a biofilm is an important factor in BV, but there is still much more research to be done to unravel the exact mechanism of biofilm development in this condition. In our research, we have focused on two prominent bacteria: *G. vaginalis* and *A. vaginae*. However, BV is a polymicrobial condition, and therefore it is necessary to study other BV-associated bacteria as well and to define their role in the development of a BV-associated biofilm. Thanks to state-of-the-art molecular techniques, the spectrum of microorganisms involved in BV has been described quite well. However, knowledge about their involvement in the BV biofilm and their interactions is lacking. FISH can elucidate the architecture of the biofilm and is useful to identify the bacteria involved in this biofilm, but designing and validating a large set of probes specific for every bacterium involved can be challenging.

An improved, well-designed in vitro three-dimensional human vaginal epithelial cell model would be an asset to study the development of a biofilm on the vaginal epithelium. This cell model should ideally consist of human vaginal cells that grow into stratified squamous multilayer epithelium with tight junctions and integration of immune cells as demonstrated in the cell culture insert multilayer model [213, 214] and the production of mucus as shown in the bioreactor derived 3D cell model [215, 216]. The differentiation of the cell culture should be relative relatively fast and it should remain viable long enough to allow the development of bacterial biofilm. With such a cell model, the adhesion capacity and the interactions between BV-associated bacteria could be studied, but it could also be used to test prevention and treatment strategies. Besides the use of the cell model in studying BV-associated bacteria, it could also be used to investigate the role of the health-associated *Lactobacillus* species. We did not detect a lactobacilli biofilm using FISH on the vaginal slides, but there is still uncertainty about biofilm development by lactobacilli in the vagina, which they could use as a way of competing with the BV-associated bacteria for adherence. The cell model would enable investigating the role of different vaginal *Lactobacillus* species in the development of vaginal biofilm, as well as the potential of lactobacilli to prevent and resolve the BV-associated biofilm , or recolonise the epithelium after treatment.

Furthermore, the findings in this thesis may be used to fine-tune the diagnosis of BV. We have already confirmed that the biofilm is of great significance in BV, and that it can easily be spotted as "clue cells" using microscopy after Gram staining or in a wet mount. Therefore, it would be advisable to always include the evaluation of clue cells while assessing vaginal slides according to the Nugent score. In research settings, it will be important to use molecular techniques such as qPCR to quantify the presence of not only *G. vaginalis*, since this bacterium is not specific enough for the diagnosis of BV. Other BV-associated bacteria, with special attention to *A. vaginae*, should be taken into account

as well. Ideally, a combination of the presence of BV-associated bacteria and absence of health-associated lactobacilli should be used to detect BV. This method was proposed by Jespers et al. [174] and would ideally be engineered as a rapid quantitative molecular assay.

In addition, the use of sialidase by *G. vaginalis* should be explored further as well. This could be done using the above proposed three-dimensional in vitro model, by comparing the biofilm-developing potential of a sialidase-producing *G. vaginalis* and a "knock-out" mutant of this strain. When straightforward evidence for the need of sialidase for adherence to the epithelial cells is obtained, this knowledge could be used in the development of treatment and prevention methods (e.g. sialidase inhibitors) for BV, and ultimately other bacterial infections.

Finally, it will be absolutely necessary to further investigate the effect of the vaginal microbiome and vaginal biofilm on intravaginal rings and vice versa. Intravaginal rings have already been used for years to deliver active products such as hormones. Moreover, their efficacy as a delivery mode for HIV prevention and treatment of STIs and vaginal conditions is currently being studied. Yet, we still do not know the effect of a foreign body on the vaginal microbiome: will the vaginal condition deteriorate after long-term exposure to a potential biofilm-attracting device or will the *Lactobacillus* dominated microbiome remain balanced after all? Moreover, it will be necessary to investigate the effect of the biomass buildup on the product-dispensing capacity of the intravaginal rings: hampering of release would be very unfavourable for the correct action of therapeutic agents in the rings.

In the end, BV remains a complicated and atypical condition and there are still several challenges to be dealt with. In this thesis, we have contributed to solving part of the puzzle, but joint efforts will be needed to uncover all bacteria involved in the BV-associated biofilm and use this information to design new and better diagnostic tools and methods of treatment or to optimise the current ones.

5

Summary

The vagina plays a major part in the likelihood of conception and the probability of a full term delivery, and in the protection against intruding pathogens. The state of the vaginal environment is very important for a positive outcome. The most favourable state of the vaginal microbiome is one in which the lactobacilli are abundant, and in which the vagina is protected and kept moist by cervicovaginal fluid. Conversely, a considerably unfavourable microbiome state is one where the beneficial lactobacilli are being overruled by other, more pathogenic, anaerobic bacteria, as seen in bacterial vaginosis (BV).

BV is the most prevalent vaginal disorder worldwide, and is associated with an entire array of serious gynaecological and obstetric complications and with an increased incidence of sexually transmitted infections (STIs). At present, little knowledge about the exact aetiology of BV is available, but the typical spectrum of bacteria that overgrow the vaginal microbiome in BV has been reasonably well-described. The two main players in this vaginal microbiome imbalance are *Gardnerella vaginalis*, often found in the *Lactobacillus*-dominated microbiome as well, and *Atopobium vaginae*. In addition, the presence of a bacterial biofilm in BV has been established to a certain extent, and is reflected by the presence of clue cells, epithelial cells covered with bacteria, in the vaginal fluid. This bacterial biofilm is a polymicrobial group of sessile bacteria adhering to the vaginal epithelium sticking together in a self-produced mucous matrix. Being part of a biofilm results in an enhanced tolerance to antimicrobial treatment and to the host immune system.

In this thesis, we delved into the role of *G. vaginalis* and *A. vaginae* in the BV biofilm. We designed new probes for fluorescence in situ hybridisation (FISH) to visualise and identify *G. vaginalis* and *A. vaginae* in the biofilm using vaginal samples on microscopy slides. In chapter 4.2, we described the probe design and method validation.

Chapter 4.3 reported on the application of this technique on a large set of vaginal slides. Using FISH, we were able to confirm and visualise the dominant presence of *G. vaginalis*

in the biofilm. Further, we reported on the symbiosis between *A. vaginae* and *G. vaginalis*: the probability of having BV increased when *A. vaginae* was present in the *G. vaginalis*-dominated biofilm.

Because *G. vaginalis* can also be detected in the health-associated lactobacilli-dominated microbiome, we explored the role of *G. vaginalis'* sialidase gene in chapter 4.4. Sialidase is an enzyme that can increase the virulence of *G. vaginalis*, for example by facilitating adherence to the epithelium after cleaving off the sialic acid on the glycans of mucous epithelial membranes. In chapter 4.4, we established that the presence of the *G. vaginalis* sialidase gene, as measured by quantitative polymerase chain reaction (qPCR) in the vaginal specimens, was associated with a BV diagnosis and with the existence of a *G. vaginalis*-dominated biofilm. The impact of the polymicrobial biofilm and the ability of *G. vaginalis* to produce sialidase are important findings which could lead to improved techniques for BV diagnosis. Furthermore, these results should be taken into account while designing and developing new strategies for BV treatment and prevention.

The existence of a vaginal biofilm is of importance in relation to medical devices applied vaginally such as vaginal rings. Currently, vaginal rings are available for contraception and treatment of vaginal atrophy. Moreover, rings are also being developed and studied for the controlled release of compounds to prevent and treat STIs. Limited information is available on the colonisation of these vaginal rings when used continuously for a period of three months or more. In chapter 4.5, we demonstrated that it was common for vaginal rings to be covered with biomass consisting of vaginal epithelial cells and associated bacteria after three weeks use. We also showed that a higher density of this biomass on the contraceptive rings was related to: BV, a vaginal biofilm with *G. vaginalis* and *A. vaginae*, and the presence of *A. vaginae* on the contraceptive vaginal ring. More research will be needed to better understand if this biomass could have an impact on the vaginal microbiome state and, possibly, on the release of active product from the rings.

The work leading to this thesis has contributed to solving part of the complicated BV puzzle, but –as always– more research will be needed to determine the role of BV-associated bacteria to unravel the aetiology of BV.

Samenvatting

De vagina speelt een belangrijke rol in een vrouwenlichaam: ze bepaalt mee de kans op bevruchting en op het voldragen van de zwangerschap, en biedt bescherming tegen binnendringende pathogenen. De toestand van de vaginale omgeving is bepalend voor een goed resultaat. In de meest gunstige toestand van het vaginale microbioom zijn er voldoende lactobacillen aanwezig en is de vagina beschermd en bevochtigd door cervicovaginaal vocht. In de ongunstige toestand van bacteriële vaginose (BV) worden deze goede lactobacillen verdreven door pathogene anaerobe bacteriën.

BV is de meest voorkomende vaginale aandoening wereldwijd en is een risicofactor voor gynaecologische en verloskundige complicaties en kan leiden tot een verhoogde incidentie van seksueel overdraagbare aandoeningen. Op dit moment is er nog maar weinig geweten over de exacte oorzaak van deze aandoening, maar er is wel al een redelijk goede beschrijving van het typische spectrum van bacteriën die voor de overgroei in de vagina zorgen. Twee hoofdrolspelers in dit onevenwichtige microbioom zijn *Gardnerella vaginalis*, een bacterie die ook dikwijls in het *Lactobacillus*-gedomineerde microbioom te vinden is, en *Atopobium vaginae*. Bovendien hebben we voldoende aanwijzingen dat er een bacteriële biofilm aanwezig is in BV. Dit kan immers gestaafd worden met de aanwezigheid van "clue-cellen", epitheelcellen bedekt met bacteriën, in het vaginale vocht. Deze bacteriële biofilm bestaat uit verschillende soorten onbeweeglijke bacteriën die zich vasthechten aan het vaginale epitheel en samengehouden worden in een zelf-geproduceerde slijmerige matrix. Door deel uit te maken van een biofilm, zijn deze bacteriën minder gevoelig voor antibiotica en het immuunsysteem van de gastheer.

In deze thesis hebben we de rol van *G. vaginalis* en *A. vaginae* in de BV-biofilm uitgespit. We hebben nieuwe probes ontwikkeld voor fluorescentie in situ hybridisatie (FISH) om *G. vaginalis* en *A. vaginae* te identificeren en in beeld te brengen als deel van de biofilm. In hoofdstuk 4.2 hebben we de ontwikkeling van de probes en de validatie van de techniek beschreven.

In hoofdstuk 4.3 rapporteren we het gebruik van deze techniek voor een grote set van vaginale stalen. Door middel van visualisatie met behulp van FISH hebben we kunnen bevestigen dat *G. vaginalis* dominant aanwezig is in de biofilm. Verder hebben we ook aangetoond dat er een symbiose is tussen *A. vaginae* en *G. vaginalis*: wanneer *A. vaginae* deel uitmaakt van de *G. vaginalis*-gedomineerde biofilm is een BV-diagnose waarschijnlijker.

G. vaginalis kan echter ook gedetecteerd worden in het door *Lactobacillus* gedomineerde gezonde microbioom. Daarom deden we onderzoek naar de rol van het *G. vaginalis* sialidase-gen in hoofdstuk 4.4. Het enzyme sialidase kan de virulentie van *G. vaginalis* verhogen, bijvoorbeeld door het afknippen van het siaalzuur van glycanen op het slijmerige epitheel, waardoor de aanhechting aan het vaginale epitheel vergemakkelijkt wordt. In hoofdstuk 4.4 tonen we aan dat de aanwezigheid van dit *G. vaginalis* sialidase-gen, gemeten met kwantitatieve polymerase chain reaction (qPCR) in vaginale stalen, correleert met BV-diagnose en met het voorkomen van een door *G. vaginalis* gedomineerde biofilm. De impact van deze polymicrobiële biofilm en het vermogen van *G. vaginalis* om sialidase te produceren zijn twee belangrijke bevindingen die kunnen leiden tot verbeterde technieken voor BV-diagnose. Deze resultaten zouden ook in gedachten moeten gehouden worden bij het ontwikkelen van nieuwe strategieën voor de behandeling en preventie van BV.

Een vaginale biofilm kan ook belangrijke gevolgen hebben bij het gebruik van medische hulpmiddelen die ingebracht worden in de vagina, zoals vaginale ringen. Op dit moment worden vaginale ringen gebruikt voor contraceptie en de behandeling van vaginale atrofie. Maar dit soort ringen wordt ook ontwikkeld en bestudeerd voor de gecontroleerde vrijgave van medicatie om seksueel overdraagbare aandoeningen te voorkomen en behandelen. Er is slechts beperkte informatie beschikbaar in verband met de kolonisering van deze vaginale ringen wanneer ze continu gebruikt worden voor een periode van minstens drie maanden. In hoofdstuk 4.5 tonen we dat het niet uitzonderlijk is dat vaginale ringen na drie weken gebruik begroeid waren met een biomassa die bestond uit vaginale epitheelcellen en hiermee geassocieerde bacteriën. Verschillende factoren correleerden met een hogere densiteit van de biomassa op de ring: BV, een vaginale biofilm bestaande uit *G. vaginalis* en *A. vaginae*, en de aanwezigheid van *A. vaginae* op de contraceptie-ringen. Er is nog meer onderzoek nodig om te weten of de biomassa een impact heeft op de toestand van het vaginale microbioom en op de vrijgave van medicatie uit de ringen.

Het werk dat tot deze thesis geleid heeft, heeft een stukje van de gecompliceerde BV-puzzel opgelost, maar er is –zoals altijd– nog meer onderzoek nodig om de rol van BV-geassocieerde bacteriën te bepalen om de etiologie van BV op te helderen.

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Appendix: Methods

A1.1 Vaginal samples

A1.1.1 Fluorescence in situ hybridisation

Preparation of vaginal samples for FISH One cotton swab was brushed against the lateral walls of the vagina. The cotton swab was immediately rolled (thinly and evenly) on a Superfrost Plus slide (Menzel-Gläser). This slide was heat-fixed by passing twice through a flame. The Superfrost Plus slides were stored for maximum six months and shipped to ITM at room temperature and fixed for a minimum of 12 hours at ITM, submerged in Carnoy solution (6:3:1, ethan ol:chloroform:glacial acetic acid).

FISH for *A. vaginae*, *G. vaginalis* and *Lactobacillus* genus Multiplex hybridisation was performed on a Superfrost Plus slide in a 5 mm^2 quadrant hybridisation area marked with a PAP pen (Sigma Aldrich, St. Louis, USA), a liquid-repellent slide marker. The slide was covered with a cover slip after addition of a hybridisation buffer that contained 200 nM of each probe: species-specific probes for *A. vaginae* (AtoITM1), *G. vaginalis* (Gard162), *Lactobacillus* genus (Lac663) and the broad-range BacUni-1 probe (Table 1. The hybridisation solution consisted of:

- 10% (wt/vol) dextran sulphate (Sigma Aldrich)
- 10 mM NaCl (Merck KGaA, Darmstadt, Germany)
- 2% (vol/vol) formamide (Merck KGaA)
- 0.1% (wt/vol) sodium pyrophosphate (Sigma Aldrich)
- 0.2% (wt/vol) polyvinylpyrrolidone (Sigma Aldrich)

- 0.2% (wt/vol) Ficoll (Sigma Aldrich)
- 5 mM disodium EDTA (Merck KGaA)
- 0.1% (vol/vol) Triton X-100 (Acros Organics, Geel, Belgium)
- 50 mM Tris-HCl at pH 7.5 (Sigma Aldrich)

The slides were incubated in a hybridisation oven (Shake 'N Bake, Boekel Scientific, Feasterville, Pennsylvania) in humid conditions, which were achieved by adding a small tray of water, at 60 °C for 60 minutes. After the slides were rinsed with double-distilled (dd) H_20, they were immersed in a 50 ml tube containing a washing solution containing 5 mM Tris base, 15 mM NaCl and 0.1% (vol/vol) Triton X-100 (at pH 10) for 15 min at 60 °C on the rocking shelves of the hybridisation oven. After this washing step, the slides were rinsed again with ddH20 and air-dried in the dark at room temperature. Subsequently, the slides were counterstained with 6-diamidine-2-phenylindole dihydrochloride (DAPI) (Serva, Heidelberg, Germany), a DNA-intercalating agent that stains the chromosomes of both prokaryotic and eukaryotic cells, for 5 minutes at room temperature in the dark and rinsed with ddH_20. Before imaging, the slides were air-dried at room temperature in the dark.

Table 1: Probe specifications

Name	Target	Probe sequence (5'-3')	Tm (°C)	%GC	Reference
AtoITM1	*Atopobium vaginae*	Alexa488-OO-CTC-CTG-ACC-TAA-CAG-ACC	66	55.6	[209]
Lac663	*Lactobacillus* genus	Alexa488-OO-ACA-TGG-AGT-TTC-CCA-CT	75.4	47.1	[205]
Gard162	*Gardnerella vaginalis*	Alexa647-OO-CAG-CAT-TAC-CAC-CCG	61	60.0	[204]
BacUni-1	Eubacteria	Alexa555-CTG-CCT-CCC-GTA-GGA	64	66.7	[416]

Microscopic analysis of hybridised samples The hybridised samples were stored in the dark at room temperature for a maximum of one week before microscopic observation using confocal laser scanning microscopy (CLSM) (LSM700, Zeiss, Oberkochen, Germany). The microscope operates with four stable, solid-state lasers at wavelengths of 405 to 639 nm, and is therefore able to detect all three fluorescently labelled probes and the DAPI stain at once. The sample was first scanned at 100X magnification to evaluate the homogeneity of the sample (objective: EC Plan-Neofluar 10x/0.30 Ph1 M27), before individual bacteria were identified at 400X magnification (objective: Plan-Apochromat 40x/1.3 Oil Ph3 M27). Three different fields were evaluated. Separate scattered bacterial cells were defined as dispersed/planktonic bacteria. Aggregates of bacterial cells attached to each other or to the vaginal epithelial cells were defined as adherent bacteria forming

Table 2: Master mix composition for qPCR

Master mix	Lactobacilli	*A. vaginae*	*G. vaginalis*	*G. vaginalis* sialidase
Rotor-Gene SYBR green	12.5 µl	12.5 µl	12.5 µl	12.5 µl
10 µM forward primer	2 µl	2.5 µl	2.5 µl	
10 µM reverse primer	2 µl	2.5 µl	2.5 µl	
5 µM forward primer				0.75 µl
5 µM reverse primer				0.75 µl
RNAse free water	3.5 µl	2.5 µl	2.5 µl	6 µl

a biofilm. The species-specific signal was considered positive only if it had a positive counterpart in the DAPI stain and if it displayed a positive signal simultaneously with the broad-range probe.

A1.1.2 Quantitative polymerase chain reaction

Preparation of vaginal samples for qPCR Two Copan flocked swabs (Copan, Brescia, Italy) were brushed against the lateral walls of the vagina. The Copan flocked swabs were eluted by vortexing each swab for at least 15 seconds in 1.2 ml of dPBS (pH 7.4 - 1:9, PBS:saline). The two eluates were combined and divided into three aliquots, which were stored at -80 °C. The swab eluates were shipped frozen (-191 °C) in a dry shipper to the ITM.

qPCR for *A. vaginae*, *G. vaginalis*, *G. vaginalis* sialidase and *Lactobacillus* genus DNA was extracted from 250 µl of the eluate using the Abbott m2000sp automated extraction platform (Abbott, Maidenhead, UK), according to the manufacturer's instructions. The volume of 200 µl DNA extract was stored at -80 °C until testing. qPCR was performed for each bacterial species in a separate reaction, to avoid competition between the primers. The 25 µl PCR mixture contained 12.5 µl Rotor-Gene SYBR Green RT-PCR Master mix (Qiagen, Venlo, the Netherlands), 5 µl DNA extract, 1 µM of *A. vaginae* or 1 µM of *G. vaginalis* or 1 µM of *G. vaginalis* sialidase or 0.8 µM *Lactobacillus* genus forward and reverse primers (Integrated DNA Technologies, Leuven, Belgium) and RNase-free water provided with the Rotor-Gene SYBR Green PCR kit (Table 2. The primers targeting the 16S rRNA and cycling conditions for the separate species are listed in Table 3.

Quantification was done using standard curves, constructed using DNA extracts from *A. vaginae* (CCUG 38953T), *G. vaginalis* (LMG 7832T) or a mixture of lactobacilli (*L. crispatus* LMG 9479, *L. iners* LMG 18914, *L. gasseri* LMG 9203, *L. jensenii* LMG 6414, *L. vaginalis* LMG 12981) , grown at 35 °C ± 2 °C on Columbia agar base (Becton Dickinson) + 5% horse blood, under anaerobic conditions. DNA concentrations were determined

Table 3: Primers and conditions for qPCR

Target	Primers	Cycling conditions	Reference
Lactobacillus genus	F-LBF: 5'-ATGGAAGAACACCAGTGGCG-3' R-LBR: 5'-CAGCACTGAGAGGCGGAAAC-3'	15 min 95 °C (15 sec 95 °C, 45 sec 50 °C, 45 sec 72 °C) x 37	[193]
A. vaginae	ATOVAGRT3Fw: 5'GGTGAAGCAGTGGAAACACT-5' ATOVAGRT3Rev: 5'-ATTCGCTTCTGCTCGCGCA-3'	15 min 95 °C (20 sec 95 °C, 45 sec 60 °C, 45 sec 72 °C) x 45	[168]
G. vaginalis	F-GV1: 5'-TTACTGGTGTATCACTGTAAGG-3' R-GV3: 5'-CCGTCACAGGCTGAACAGT-3'	15 min 95 °C (45 sec 95 °C, 45 sec 55 °C, 45 sec 72 °C) x 50	[193]
G. vaginalis sialidase	GVSI_Forward: 5'-GACGACGGCGAATGGCACGA-3' GVSI_Reverse2: 5'-TACAAGCGGCTTTACTCTTG-3'	10 min 95 °C, (5 sec 95 °C, 10 sec 58 °C) x 45	[160]

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using NanoDrop (Thermo Fisher Scientific, Erembodegem, Belgium) and the number of genomes was calculated using the described genome sizes and G+C content of the strains. A total of six tenfold dilutions of the DNA stocks were prepared in high performance liquid chromatography (HPLC) grade water. Both the standard curve and samples were run in duplicate. The bacterial load was expressed as genome equivalents (geq)/ml, because quantification is based on cultured bacteria. The qPCR does not distinguish between living and dead bacteria, therefore geq is a better term than copies or bacteria/ml, although one geq is the equivalent of one copy or one bacterium.

A1.2 Ring samples

A1.2.1 Preparation of rings for further analysis

All rings worn by study participants were collected after removal at study visits. Each ring was cut in three equal parts immediately after removal:

- The first part for the biomass density assessment with crystal violet was submerged in 3 ml of glutaraldehyde for two weeks, transferred to 3 ml of formaldehyde, and stored at 2-8 °C until testing.
- The second part for qPCR was stored in diluted phosphate buffered saline (dPBS) (pH 7.4 - 1:9, PBS:saline) at -20 °C.
- The third part for FISH was stored in Carnoy solution (6:3:1, ethanol:chloroform:glacial acetic acid) at 2-8 °C until testing.

All samples, except for the Nugent slide, were shipped from the study site in Rwanda to the ITM in Antwerp, Belgium: the refrigerated samples were transported at room temperature, while the frozen samples were transported at -196 °C in a dry shipper.

A1.2.2 Fluorescence in situ hybridisation

First the rings were rinsed with ddH_2O. After 10 minutes of air-drying (not fully dry!), the ring-biomass was removed with tweezers, carefully without rupturing the biofilm. The biofilm was spread out on a Superfrost Plus slide (Menzel-Gläser) and passed through a flame twice to fixate the slide. A second fixation step was done by submerging the slide in Carnoy solution for 12 hours. FISH was performed as described above for the vaginal samples, but the whole fixated sample was used and flown with hybridisation buffer + probes.

A1.2.3 Quantitative polymerase chain reaction

Frozen ring parts were thawed and vortexed; using this eluate, 200 µl DNA was extracted (Abbott, Maidenhead, UK) and stored at -80 °C until testing. qPCR was performed as described above for the vaginal samples.

A1.2.4 Crystal violet assay

The biomass on contraceptive rings were stained with crystal violet to determine the optical density (OD) as a proxy for the quantity of the biomass. The ring was submerged in water, to rinse off the loose/not attached cells. Then the ring-biomass was stained by submerging in a small tube containing 3 ml crystal violet (0.1% solution) for 10 to 15 minutes. Next, the ring part was rinsed with water twice and air-dried for two hours. The dried ring-biomass staining was solubilised by submerging the ring in 3 ml of 30% acetic acid in water for 10 to 15 minutes. From this solution, 125 µl was transferred to a new micro-titre plate for OD measurement at 550 nm. OD measurements were done in duplo. An unworn ring was stained the same way as the used rings and used as the blank; the OD value of this ring (in duplo) was subtracted from all measured ODs.

Appendix: Curriculum vitae

LISELOTTE HARDY

Voorhavenlaan 50 | 9000 Ghent | Belgium
+32 (0)472/344187 | liselotte.hardy@gmail.com | 26 June 1984

Skills and strengths

Technical strengths	qPCR, FISH, light microscopy, CLSM, bacterial and cell culture
Clinical research	GCP/GLP, writing of and training in protocols and SOPs, reporting
Computer knowledge	MS Office, Mac OS X, Windows, Stata, R, LaTeX, ImageJ, Matlab
Project management	budgeting and planning, internal and external communication
Languages	excellent Dutch and English, average French

Professional experience

Antelope Diagnostics - Ghent University June 2016 - Present

Assay development/Quality management

- Development and validation of novel point-of-care assays
- Introduction quality systems for in vitro diagnostics

AZ Nikolaas - Oncology Department December 2015 - May 2016

Study coordinator/data manager

- Coordinating clinical research studies at the oncology department
- Planning, preparing, realising and evaluating ongoing and new clinical studies
- Data management and administrative tasks

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Institute of Tropical Medicine/Ghent University January 2013 - April 2015
Scientific staff

- PhD research: "Characterisation of bacterial biofilm associated with bacterial vaginosis"
- Developing and implementing Fluorescence in situ Hybridisation (FISH) and quantitative polymerase chain reaction (qPCR) to detect and identify biofilm-forming bacteria
- Set-up of vaginal epithelial cell model to study biofilm development
- Writing research papers and presenting results at national and international conferences
- Supervising master and bachelor students

Institute of Tropical Medicine November 2009 - January 2013
Project coordinator

- "MMIS study": a descriptive study on the mucosal microbiome and associated immune factors in adolescent girls in Antwerp, Integrated Project for the EC 7th Framework programme
- "Biomarkers study": a project studying safety biomarkers for the use of microbicides in Rwanda, Kenya and South Africa, funded by EDCTP
    - Writing protocols, SOPs, guidelines and manuals
    - Preparing, coordinating and implementing studies
    - Laboratory analysis: qPCR, FISH, ELISA, Luminex
    - Communication, reporting and presenting study results

Quintiles Belgium December 2007 - November 2009
Clinical Research Associate

- Start-up, monitoring and close-out of clinical studies (phase 2 and 3) according to Good Clinical Practices (GCP)
- Training in protocols and study procedures

Education

Postgraduate in Tropical Medicine and International Health February 2007
Institute of Tropical Medicine, Antwerp

Master in Biomedical Sciences June 2006
Ghent University

Bachelor in Biomedical Sciences June 2004
Hasselt University/Transnational University Limburg

Additional training

Language courses

- *Advanced academic English: English proficiency for presentations* March 2015
  University Language Center, University of Ghent
- *Writing academic papers in English* January 2015
  Linguapolis, University of Antwerp

Technical training

- *Molecular and physiological regulation of microbial biofilms* September 2014
  K.U.Leuven
- *International practical course on biofilm science* July 2014
  University of Minho, Portugal
- *Postgraduate technical workshop "Medical biofilm techniques"* August 2012
  Danish Technical University, Kopenhagen
- *PhD/Master course "Advanced microscopy and vital imaging"* June 2012
  University of Maastricht, The Netherlands

Additional training

- *Project management* January 2016
  Ghent University
- *Statistical analysis in R for health scientists* October 2015
  Ghent University
- *Specific teacher training* June 2007
  Ghent University

Conferences attended

- World STI and HIV Congress, 2015, Brisbane
- Belgian Interdisciplinary Biofilm Research meeting, 2014, Louvain-la-Neuve
- Belgian Interdisciplinary Biofilm Research meeting, 2013, Louvain-la-Neuve
- Eurobiofilms, 2013, Ghent
- Nobel conference on biofilm formation, 2013, Stockholm
- World STI and AIDS Congress, 2013, Vienna
- American Society for Microbiology conference on biofilms, 2012, Miami

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- **Hardy L**, Jespers V, Abdellati S, De Baetselier I, Mwambarangwe L, Musengamana V, van de Wijgert J, Vaneechoutte M, Crucitti T. *A Fruitful Alliance: the Synergy Between Atopobium vaginae and Gardnerella vaginalis in Bacterial Vaginosis-Associated Biofilm.* STI 2016;0:1–5.
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- Gautam R, Borgdorff H, Jespers V, Francis SC, Verhelst R, Mwaura M, Delany-Moretlwe S, Ndayisaba G, Kyongo JK, **Hardy L**, Menten J, Crucitti T, Tsivtsivadze E, Schuren F, van de Wijgert J for the Vaginal Biomarkers Study Group. *Correlates of the Molecular Vaginal Microbiota Composition of African Women.* BMC Infectious Diseases 2015 15:86.
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- **Hardy L**, Jespers V, De Baetselier I, van de Wijgert J, Crucitti T. *The Impact of Vaginal Bacterial Biofilm on Intravaginal Rings.* STI 91:Suppl 2 A53-A54. World STI and HIV Congress 2015, Brisbane. (Travel scholarship awarded)
- Jespers V, **Hardy L**, Buvé A, Loos J, Tsoumaris A, Buyze J, Crucitti T. *The Influence of Sexual Debut on Selected Vaginal, Rectal and Oral Microbiota and Vaginal Inflammatory Markers in Belgian Adolescent Girls: a Cohort Study.* STI 91:Suppl 2 A34. World STI and HIV Congress 2015, Brisbane.
- **Hardy L**. *New Insights in Biofilm Associated with Bacterial Vaginosis.* Summer School "Molecular and physiological regulation of microbial biofilms" 2014, Leuven.
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- Mwaura M, **Hardy L**, Delany-Moretlwe S, Ndayisaba G, Mandaliya K, Cools P, Verhelst R, van de Wijgert J, Crucitti T, Jespers J. *There is a Need for Multi-purpose Prevention Technologies Targeting HIV and Common Reproductive Tract Infections: Data from the Biomarkers Study Team.* 7th EDCTP forum 2014, Berlin.
- **Hardy L**. *Microbiome Results from Biomarkers Trials.* CHAARM annual consortium meeting 2014, Camogli.
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Appendix: Papers as published

PLOS ONE

RESEARCH ARTICLE

Unravelling the Bacterial Vaginosis-Associated Biofilm: A Multiplex *Gardnerella vaginalis* and *Atopobium vaginae* Fluorescence *In Situ* Hybridization Assay Using Peptide Nucleic Acid Probes

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Abstract

Bacterial vaginosis (BV), a condition defined by increased vaginal discharge without significant inflammation, is characterized by a change in the bacterial composition of the vagina. *Lactobacillus* spp., associated with a healthy vaginal microbiome, are outnumbered by BV-associated organisms. These bacteria could form a polymicrobial biofilm which allows them to persist in spite of antibiotic treatment. In this study, we examined the presence of *Gardnerella vaginalis* and *Atopobium vaginae* in vaginal biofilms using Peptide Nucleic Acid (PNA) probes targeting these bacteria. For this purpose, we developed three new PNA probes for *A. vaginae*. The most specific *A. vaginae* probe, AtoITM1, was selected and then used in an assay with two existing probes, Gard162 and BacUni-1, to evaluate multiplex FISH on clinical samples. Using quantitative polymerase chain reaction (qPCR) as the gold standard, we demonstrated a sensitivity of 66.7% (95% confidence interval: 54.5% - 77.1%) and a specificity of 89.4% (95% confidence interval: 76.1% - 96%) of the new AtoITM1 probe. FISH enabled us to show the presence of a polymicrobial biofilm in bacterial vaginosis, in which *Atopobium vaginae* is part of a *Gardnerella vaginalis*-dominated biofilm. We showed that the presence of this biofilm is associated with high bacterial loads of *A. vaginae* and *G. vaginalis*.

Introduction

Bacterial vaginosis (BV), a condition characterized by increased vaginal discharge without significant inflammation, is highly prevalent in women of reproductive age. It increases the risk

role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Funding was received by TC from European and Developing Countries Clinical Trials Partnership: SP.2011.41304.043, http://www.edctp.org/; by VJ from Combined Highly Active Anti-Retroviral Microbicides under EU FP7: 242135, http://cordis.europa.eu/fp7/home_en.html; and by VJ from Dormeur Investment Service Ltd. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

for acquisition and transmission of sexually transmitted infections, including HIV, and is associated with preterm birth in pregnant women [1–2]. BV is a dysbiotic condition of unknown etiology and is characterized by a change in the microbial composition of the vagina. *Lactobacillus* spp., associated with a healthy vaginal microbiome, are outnumbered by an array of BV-associated organisms including *Gardnerella vaginalis* [1–3]. However, several studies suggest that the mere presence of *G. vaginalis* is not sufficient for the diagnosis of BV. Indeed, *G. vaginalis* is also present in 50% to 70% of women without BV according to Nugent score [4–6]. *G. vaginalis* expresses various virulence factors such as vaginolysin [7] and sialidase [8]. It can also produce a biofilm [9], thereby increasing its tolerance to lactic acid and hydrogen peroxide produced by lactobacilli [10,11] and to antimicrobial treatment [12,13]. Furthermore, it has been suggested that its adherence and biofilm-forming capacities allow *G. vaginalis* to initiate the colonization and scaffolding of the vaginal epithelium to which other species can attach subsequently [14,15].

As was first shown a decade ago, *Atopobium vaginae* is one of the many other species that are characteristic of BV [16–20]. In one study, *A. vaginae* was detected in 80% of samples testing positive for *G. vaginalis* and made up 40% of the total biofilm mass dominated by *G. vaginalis* [9]. This association was confirmed in a study by Bradshaw et al. [21]: 93% of samples containing *A. vaginae* also contained *G. vaginalis*, whereas only 10% tested positive for *G. vaginalis* when *A. vaginae* was absent [22]. In contrast to *G. vaginalis*, *A. vaginae* is rarely part of the healthy vaginal microbiome and is considered a more specific marker of BV than *G. vaginalis* [17,21,23].

It is postulated that a biofilm provides bacteria with a competitive advantage over planktonic bacteria and that polymicrobial biofilms may offer additional advantages over single-species biofilms. Mechanisms that have been described in previous studies include metabolic cooperation, increased resistance to antibiotics or host immune responses [24] and an enlarged gene pool with more efficient sharing of genetic material compared to mono-species biofilms [25]. Polymicrobial coexistence is the dominant form in environmental biofilms, but is also prominent in the human body [24]. A well-known example is dental plaque: anaerobic bacteria, which are sensitive to oxygen, can survive and persist under the aerobic conditions in the oral cavity due to the consummation of oxygen by aerobic bacteria in the dental biofilm [26].

On the basis of these previous findings, we hypothesize that a polymicrobial biofilm consisting of *A. vaginae* and *G. vaginalis* and other bacteria not discussed in this study may serve as a marker of BV. Thus, better visualization of the structure of vaginal biofilms and identification of the bacterial components of the biofilm may contribute to better understanding of BV. To study the role of *A. vaginae* and *G. vaginalis* in BV, we designed and evaluated the performance of fluorescence *in situ* hybridization (FISH) with peptide nucleic acid (PNA) probes for *A. vaginae* and *G. vaginalis*. Three new PNA probes were designed for *A. vaginae*. The most specific *A. vaginae* probe was selected, and subsequently used together with a PNA probe that had been described for the detection of *G. vaginalis* and a positive control probe that detects a broad range of bacteria in order to evaluate the multiplex FISH on clinical samples.

Materials and Methods

Design of PNA probes

PNA probes targeting the bacterial 16S rRNA were synthesized by Panagene (Daejeon, South Korea). A fluorescent label was attached using a double 8-amino-3,6-dioxaoctanoic acid (AEEA) linker at the N terminus. We evaluated the performance of three new PNA probes for *A. vaginae*: Ato291, previously described as a DNA FISH probe [9,27] and AtoITM1 and AtoITM2, which we developed using the Applied Biosystems PNA designer software (http://

Table 1. Probe specifications.

Name	Target	Probe sequence (5'-3')	Tm (°C)	%GC	Reference
AtoITM1	*A. vaginae*	Alexa488-OO-CTC-CTG-ACC-TAA-CAG-ACC	66	55.6	Newly designed, based on Burton et al. [28]
AtoITM2	*A. vaginae*	Alexa488-OO-GCG-GTY-TGT-TAG-GTC-AGG	72	58.3	Newly designed, based on Fredricks et al. [29]
Ato291	*A. vaginae*	Alexa488-OO-GGT-CGG-TCT-CTC-AAC-CC	68	60.0	Newly designed, based on Harmsen et al. [27]
Gard162	*G. vaginalis*	Alexa647-OO-CAG-CAT-TAC-CAC-CCG	61	60.0	Machado et al. [30]
BacUni-1	Eubacteria	Alexa555-CTG-CCT-CCC-GTA-GGA	64	66.7	Perry-O'Keefe et al. [32]

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www6.appliedbiosystems.com/support/pnadesigner.cfm), based on sequences of species-specific PCR primers from Burton [28] for AtoITM1 and from Fredricks [29] for AtoITM2. The probes met the following criteria: purine content was limited to less than 60%; a maximum of four purines in a purine-stretch and a maximum of three guanines in a guanine-stretch were allowed; and self-complementarity was absent, considering that PNA/PNA interactions are stronger than PNA/DNA interactions. For the detection of *G. vaginalis*, a previously described probe, Gard162 [30,31], was used. The broad-range bacterial probe, BacUni-1, previously designed [32] as a modified version of the broad-range eubacterial DNA probe [33], was used as a positive control. The probe specifications are listed in Table 1.

Bacterial culture techniques for evaluation performance PNA probes

The performance of the PNA probes was evaluated using clinical isolates, obtained from the collections of the Institute of Tropical Medicine (ITM) and Ghent University. The five most frequently detected *Lactobacillus* species [3,34], representing the non-BV microbiome, were chosen as a negative control to assure that the probes would not cross-hybridize with the normal microbiome (Table 2). Furthermore we selected the most frequent BV-associated bacteria (8 *A. vaginae* strains, 5 *G. vaginalis* strains) as a negative control for *G. vaginalis* and *A. vaginae* respectively in addition to *2 Prevotella melaninogenica* strains (Table 2). This small test panel is a limitation of the study and more expansive testing will be required to assure that there is no cross-reactivity with other bacteria. The strains from frozen stocks in skimmed milk (-80°C) were cultured on Columbia agar base (Becton Dickinson Biosciences, Erembodegem, Belgium) + 5% horse blood and grown under anaerobic conditions (10% hydrogen, 10% carbon dioxide and 80% nitrogen), using an anaerobic incubator (Whitley DG250) at 37°C for 48 h and bacteria were streaked onto fresh plates every 48–72 hours. To determine the limit of detection, FISH was performed using the AtoITM1, Gard162 and BacUni-1 probe on serial tenfold dilutions using the fixative used throughout the study: Carnoy solution (6:3:1, ethanol:chloroform:glacial acetic acid [35,36]). Carnoy solution was chosen as a fixative solution because of its proven effectiveness in the stabilization of and minimal shrinkage in tissue structure [36,37]. The concentration of the dilutions was estimated based on the turbidity of the sample compared to McFarland Standards (Bio-Merieux SA, Marcy l'Etoile, France) and ranged from $1.2x10^9$ to $1.2x10^3$ cells per ml. The suspensions were vortexed briefly and 5 μl was spotted into the hybridization chamber; a 5 mm^2 area marked with a PAP Pen (Sigma Aldrich, St. Louis, USA) that creates a hydrophobic border on a Superfrost Plus slide (Menzel-Gläser, Braunschweig, Germany). The spotted suspensions were dried before performing FISH. Experiments were performed in duplicate.

Clinical samples

Ethics statement. Vaginal samples were collected from 119 women participating in a clinical trial in Rwanda studying the vaginal microbiome and acceptability of a contraceptive ring (S1 Protocol) (the 'Ring Plus' study, ClinicalTrials.gov identifier NCT01796613) (data analysis on-

Table 2. Specificity testing in duplicate of PNA probes using cultured bacteria. The signal was considered positive if it had a positive counterpart in the DAPI stain and displayed a positive signal simultaneously with the broad-range probe. The signal was considered negative if no signal was seen with the species-specific probe.

Species	Strain	AtoITM1	AtoITM2	Ato291	Gard162	BacUni-1
Atopobium vaginae	CCUG 38953^T	+[1]	+	+	-[2]	+
Atopobium vaginae	UG080499	+	+	+	-	+
Atopobium vaginae	UG071164	+	+	+	-	+
Atopobium vaginae	UG020349	+	+	+	-	+
Atopobium vaginae	UG160373	+	+	+	-	+
Atopobium vaginae	UG550940	+	+	+	-	+
Atopobium vaginae	UG030313	+	+	+	-	+
Atopobium vaginae	UG030312	+	+	+	-	+
Gardnerella vaginalis	UG860108	-	-	-	+	+
Gardnerella vaginalis	UG030406	-	-	-	+	+
Gardnerella vaginalis	UG860107	-	+	+	+	+
Gardnerella vaginalis	LMG 7832^T	-	+	+	+	+
Gardnerella vaginalis	UG030407	-	+	+	+	+
Lactobacillus iners	LMG 18914^T	-	-	+	-	+
Lactobacillus vaginalis	LMG 12891^T	-	-	+	-	+
Lactobacillus jensenii	LMG 6414^T	-	-	+	-	+
Lactobacillus crispatus	LMG 9479^T	-	-	+	-	+
Lactobacillus gasseri	LMG 9203^T	-	-	+	-	+
Prevotella melaninogenica	UG160361	-	-	+	-	+
Prevotella melaninogenica	UG040818	-	-	-	-	+

[1](+) Presence of hybridization

[2](-) Absence of hybridization.

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going) [38]. Participants were between 18 and 35 years old and provided written informed consent for participation in the study. The Ring Plus study and consent procedure were approved by the Rwanda National Ethics Committee, Rwanda; the Institutional Review Board of the ITM Belgium; and the ethics committee of the University Teaching Hospital in Antwerp, Belgium.

Vaginal sample collection and preparation. Vaginal sampling was carried out by the study clinician as part of the study procedures. Two Copan flocked swabs (Copan, Brescia, Italy) and one cotton swab were brushed against the lateral walls of the vagina. The cotton swab was immediately rolled on a Superfrost Plus slide (Menzel-Gläser) which was heat-fixed by passing twice through a flame. The Superfrost Plus (Menzel-Gläser) slides were stored for maximum six months and shipped to ITM at room temperature and fixed for a minimum of 12 hours at ITM, submerged in Carnoy solution [35,36]. The Copan flocked swabs were eluated by vortexing each swab for at least 15 seconds in 1.2 ml of diluted phosphate buffered saline (PBS) (pH 7.4 - 1:9, PBS:saline). The two eluates were combined and divided into three aliquots, which were stored at -80°C. The swab eluates were shipped frozen (-191°C) in a dry shipper to the ITM to determine the total bacterial load of *A. vaginae* and *G. vaginalis* by means of quantitative real-time polymerase chain reaction (qPCR).

Urine sample collection and preparation. According to an earlier described procedure [39], first-void urine was collected by the participants and 2 ml was transferred immediately to a 15 ml tube containing 2 ml of Carnoy solution. The sample was fixed overnight and after centrifugation (10 minutes at 3200 g), the supernatant was decanted and the pellet was treated for a second time with 0.75 μl of Carnoy solution. The samples were stored between 2–8°C and

shipped at room temperature to the ITM. Before applying FISH, the urine samples were vortexed briefly and 5 μl was spotted into the hybridization chamber on a Superfrost Plus slide (Menzel-Gläser).

Quantitative PCR for quantification of bacteria in vaginal samples. DNA was extracted from 250 μl of the vaginal swab eluate using the Abbott m2000sp automated extraction platform (Abbott, Maidenhead, UK), according to the manufacturer's instructions. The volume of 200 μl DNA extract was stored at –80°C until testing. qPCR was performed for each bacteria species separately, to avoid competition between the primers. The 25 μl PCR mixture contained 12.5 μl Rotor-Gene SYBR Green RT-PCR Master mix (Qiagen, Venlo, the Netherlands), 5 μl DNA extract, 0.5 μM of *A. vaginae* or 1 μM of *G. vaginalis* forward and reverse primers (Integrated DNA Technologies, Leuven, Belgium) and RNase-free water provided with the Rotor-Gene SYBR Green PCR kit. The primers for *A. vaginae* and *G. vaginalis* and the amplification reactions (Rotor Gene Q MDx 5 plex, Qiagen) have been described before [34].

Quantification was done using standard curves, constructed using DNA extracts from *A. vaginae* (CCUG 38953^T) and *G. vaginalis* (LMG 7832^T), grown at 35°C ± 2°C on Columbia agar base (Becton Dickinson) + 5% horse blood, under anaerobic conditions. DNA concentrations were determined using NanoDrop (Thermo Fisher Scientific, Erembodegem, Belgium) and the number of genomes was calculated using the described genome sizes and G+C content of the strains. A total of six tenfold dilutions of the DNA stocks were prepared in high performance liquid chromatography (HPLC) grade water. Both the standard curve and samples were run in duplicate. The bacterial load was expressed as genome equivalents (geq)/ml.

PNA FISH procedure

Multiplex hybridization was performed on a Superfrost Plus slide (Menzel-Gläser) in a 5 mm² quadrant hybridization area marked with a PAP pen (Sigma Aldrich, St. Louis, USA), a liquid-repellent slide marker. The slide was covered with a cover slip after addition of a hybridization buffer that contained 200 nM of each probe: species-specific probes for *A. vaginae* (AtoITM1 or AtoITM2 or Ato291) and *G. vaginalis* (Gard162), and the broad-range BacUni-1 probe. The hybridization solution consisted of 10% (wt/vol) dextran sulphate (Sigma Aldrich), 10 mM NaCl (Merck KGaA, Darmstadt, Germany), 2% (vol/vol) formamide (Merck KGaA), 0.1% (wt/vol) sodium pyrophosphate (Sigma Aldrich), 0.2% (wt/vol) polyvinylpyrrolidone (Sigma Aldrich), 0.2% (wt/vol) Ficoll (Sigma Aldrich), 5 mM disodium EDTA (Merck KGaA), 0.1% (vol/vol) Triton X-100 (Acros Organics, Geel, Belgium) and 50 mM Tris-HCl at pH 7.5 (Sigma Aldrich).

The slides were incubated in a hybridization oven (Shake 'N Bake, Boekel Scientific, Feasterville, Pennsylvania) in humid conditions, which were achieved by adding a small tray of water, at 60°C for 60 minutes. After the slides were rinsed with double-distilled (dd) H_20, they were immersed in a washing solution containing 5 mM Tris base, 15 mM NaCl and 0.1% (vol/vol) Triton X-100 (at pH 10) for 15 min at 60°C on the rocking shelves of the hybridization oven. After this washing step, the slides were rinsed again with ddH_20 and air-dried in the dark at room temperature. Subsequently, the slides were counterstained with 6-diamidine-2-phenylindole dihydrochloride (DAPI) (Serva, Heidelberg, Germany), a DNA-intercalating agent that stains the chromosomes of both prokaryotic and eukaryotic cells, for 5 minutes at room temperature in the dark and rinsed with ddH_20. Before imaging, the slides were air-dried at room temperature in the dark.

Assessment of reproducibility of FISH

The inter-run repeatability of FISH was evaluated by comparing the outcomes of two independent FISH runs for a subset of the samples. The second hybridization was performed 6 months

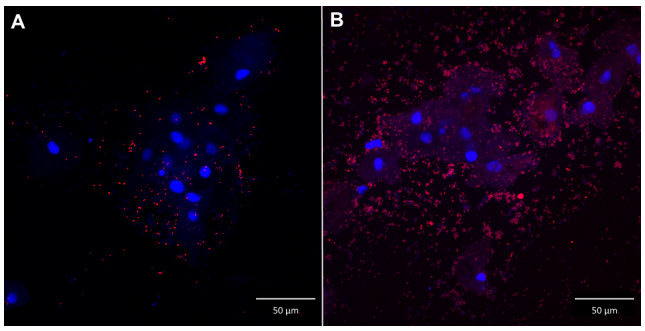

Fig 1. Dispersed bacteria versus biofilm. Confocal laser scanning images with 400x magnification of *G. vaginalis* biofilm in 2 vaginal slides (A and B) in a superimposed image: vaginal epithelial cells DAPI in blue and *G. vaginalis* specific PNA-probe Gard162 with Alexa Fluor 647 in red. A: vaginal sample with dispersed bacteria; B: vaginal sample with bacteria in biofilm.

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later on a subset of 15% of the samples (N = 17), which reflects one FISH run. We selected 17 samples showing variable results for the species-specific signal and positive for the broad-range probe. A new hybridization spot was drawn and fresh hybridization and washing buffer was used on the exact same slides used in the first FISH run. Visual inspection by confocal microscopy was performed by the same microscopist.

Microscopic analysis of hybridized samples

The hybridized samples were stored in the dark at room temperature for a maximum of one week before microscopic observation using laser scanning confocal microscopy (LSM700, Zeiss, Oberkochen, Germany). The microscope operates with four stable, solid-state lasers at wavelengths of 405 to 639 nm, and is therefore able to detect all three fluorescently labelled probes and the DAPI stain at once in one hybridization chamber. The sample was first scanned at 100x magnification (objective: EC Plan-Neofluar 10x/0.30 Ph1 M27), before individual bacteria were identified at 400x magnification (objective: Plan-Apochromat 40x/1.3 Oil Ph3 M27). Separate scattered bacterial cells were defined as dispersed bacteria (Fig 1A). Aggregates of bacterial cells attached to the vaginal epithelial cells were defined as adherent bacteria forming a biofilm (Fig 1B). The species-specific signal was considered positive only if it had a positive counterpart in the DAPI stain and if it displayed a positive signal simultaneously with the broad-range probe.

Statistical analysis

The specificity and sensitivity of FISH for vaginal slides was compared with the qPCR as a reference method. Bacterial counts were log 10 transformed before analysis. Data analysis was done using STATA13. The p-values reported for associations between the presence of bacterial species/biofilm and bacterial loads from the qPCR results originate from the non-parametric Kruskal-Wallis equality-of-populations rank test.

Results

PNA FISH probe performance on bacterial strains

The three probes specific for *A. vaginae* clearly hybridized with all eight *A. vaginae* strains tested (Table 2). However, Ato291 showed cross-hybridization with three *G. vaginalis* strains, with all *Lactobacillus* species and with one *Prevotella* strain. The newly developed AtoITM2

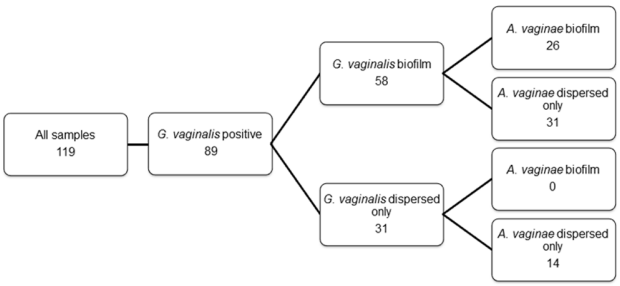

Fig 2. Distribution of samples according to FISH. Aggregates of bacterial cells attached to the vaginal epithelial cells, were defined as biofilm. Separate scattered bacterial cells, without the presence of biofilm, were defined as dispersed only bacteria.

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probe cross-hybridized with three *G. vaginalis* strains. Only AtoITM1 performed without false positive results and was selected for further evaluation on the clinical samples. The Gard162 probe was able to identify all five *G. vaginalis* test strains and showed no cross-hybridization with any of the other 15 species tested (Table 2). All bacterial strains tested hybridized with the broad-range BacUni-1 probe. According to the FISH results of the serial dilutions, the limit of detection for AtoITM1, Gard162 and BacUni-1 probes was 1.2 x 10^5 cells per ml.

Detection of *A. vaginae* and *G. vaginalis* in clinical samples by PNA FISH

In a small pilot study, a total of 10 paired vaginal slides and urine samples were tested to evaluate the suitability of each type of sample for FISH analysis. Six out of 10 urine samples could not be assessed due to the low presence of vaginal epithelial cells in the urine, whereas this problem was experienced in only 2 vaginal samples. Therefore, it was decided to continue analyses on vaginal slides only.

Using the AtoITM1 PNA-probe, *A. vaginae* was visualized as dispersed entities, without the presence of biofilm, in 27/119 (22.7%) of the samples. *A. vaginae* biofilm was present in 26/119 (21.8%) samples. *A. vaginae* FISH was negative in the remaining 66/119 (55.5%) samples. PNA-FISH using Gard162 detected dispersed-only *G. vaginalis* in 31/119 (26%) samples, *G. vaginalis* biofilm in 58/119 (48.7%) samples (e.g., Fig 2) and 30/119 (25.3%) samples were negative for *G. vaginalis*. Of the 89 *G. vaginalis* FISH-positive samples (dispersed or biofilm), 36 samples (41%) were negative for *A. vaginae*. However, all samples with *A. vaginae* biofilm showed a *G. vaginalis* biofilm as well (e.g., Figs 3, 4 and 5).

Characterization of vaginal samples by qPCR

A total of 119 vaginal samples were available for qPCR analysis. *A. vaginae* was present in 72 (60%) of the samples with a mean log of 7.55 ± 1.34 geq/ml. *G. vaginalis* was detected in 95 (80%) of the samples with a mean log of 7.38 ± 1.11 geq/ml.

Performance of probes in vaginal samples

Quantitative PCR was used as the reference method for detection and quantification of *A. vaginae* and *G. vaginalis*. The signal of the species-specific probes was only considered positive if a positive counterpart was seen in the DAPI stain and with the universal BacUni-1 probe. When assessing the results with FISH probe AtoITM1 against the qPCR outcomes for *A. vaginae* for 119 vaginal samples, FISH results were false negative for 24 samples and false positive for 5 samples, resulting in a sensitivity of 66.7% (95% confidence interval (CI): 54.5% - 77.1%) and a

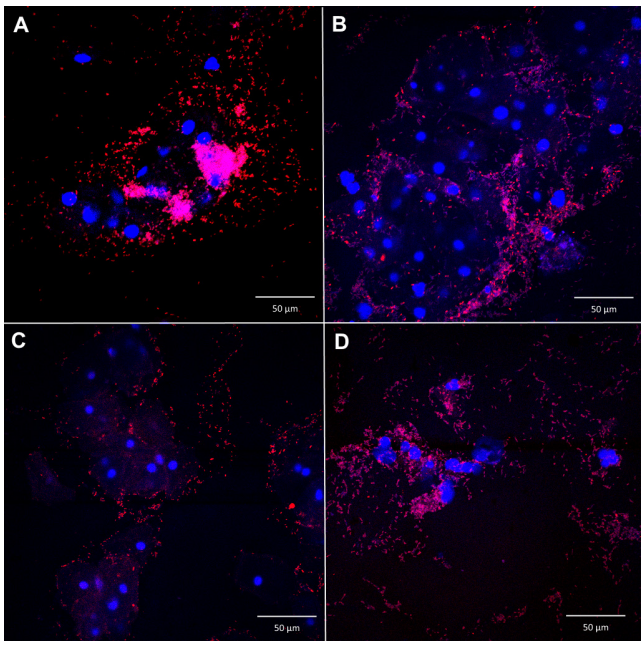

Fig 3. *G. vaginalis* biofilm. Montage of confocal laser scanning images with 400x magnification of *G. vaginalis* biofilm, negative for *A. vaginae*, in 4 vaginal samples (A-D) in a superimposed image: vaginal epithelial cells DAPI in blue and *G. vaginalis* specific PNA-probe Gard162 with Alexa Fluor 647 in red. For clarity we omitted the BacUni-1 plane; the bacteria that did not hybridize with Gard162 are visible in DAPI blue.

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specificity of 89.4% (95% CI: 76.1% - 96%) (Table 3). The mean log for the true positive samples (positive with qPCR and FISH) was 7.73 geq/ml, as compared to a mean log of 7.19 geq/ml for the false negative FISH results (p = 0.399). For Gard162, the *G. vaginalis* probe, 13 FISH results were false negative and six false positive. The sensitivity was 86.3% (95% CI: 77.4% - 92.2%) and the specificity 75.0% (95% CI: 52.9% - 89.4%) (Table 3). The mean log for the true positive results for *G. vaginalis* was 7.61 geq/ml compared to a mean log of 5.94 geq/ml for the false negative results ($p<0.001$).

Assessment of the repeatability was done using 17 samples. After the first hybridization, all samples showed a signal for the BacUni-1 probe, 5 and 9 samples out of 17 for the AtoITM1 and Gard162 probe respectively. The results of the second FISH with the BacUni-1 and Gard162 probe were in full agreement with the first run. For the AtoITM1 probe, only one sample had a different result in the second run (negative at first, but positive in the second run).

The presence of biofilm related to bacterial loads

The probability of detecting bacteria in a biofilm with FISH was higher when high ($>10^6$ geq/ml) bacterial loads for *G. vaginalis* ($p<0.001$) and *A. vaginae* ($p<0.001$) were present. The mean log of both species was highest when *A. vaginae* was part of the biofilm, compared to a biofilm of *G. vaginalis* only. *A. vaginae* biofilm was always observed together with *G. vaginalis* (Table 4).

Discussion

We set out to evaluate the performance of PNA FISH for the investigation of the vaginal polymicrobial biofilm consisting of *G. vaginalis* and *A. vaginae*. For this purpose, we evaluated

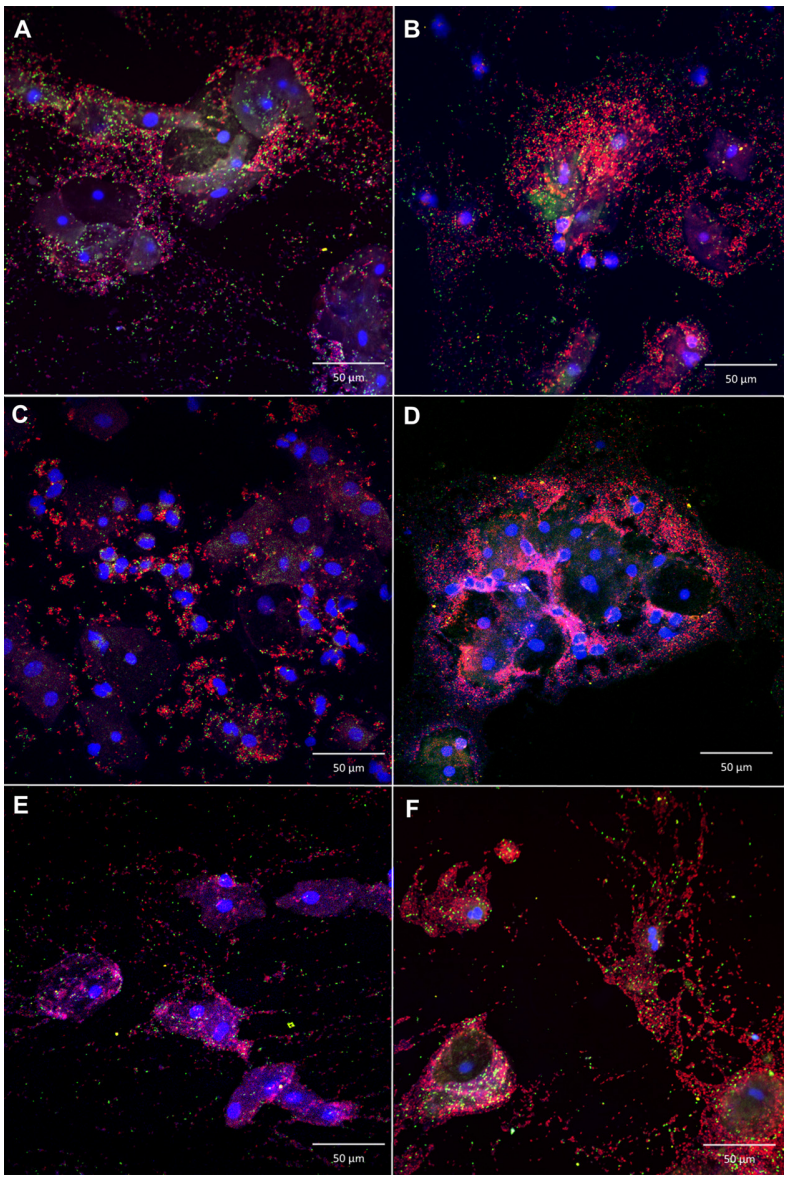

Fig 4. Superimposed image of polymicrobial biofilm of *A. vaginae* and *G. vaginalis*. Montage of confocal laser scanning images with 400x magnification of polymicrobial biofilm in 6 vaginal samples (A-F) in a superimposed image: vaginal epithelial cells DAPI in blue, *G. vaginalis* specific PNA-probe Gard162 with Alexa Fluor 647 in red and *A. vaginae* specific PNA-probe AtoITM1 with Alexa Fluor 488 in green. For clarity we omitted the BacUni-1 plane; the bacteria that are not bound by the specific probes are visible in DAPI blue.

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three newly designed *A. vaginae* PNA probes for their specificity and applied the most specific one, AtoITM1, on a range of fixed vaginal slides together with an already existing *G. vaginalis* and broad-range PNA probe.

Hybridization-based techniques such as FISH have been used in various disciplines, such as cytogenetics and microbiology, to detect the presence or absence of nucleic acid sequences. Detection of DNA and RNA is generally done using DNA probes but the use of PNA probes is increasing. PNA molecules have a neutral backbone giving them a significant advantage in low

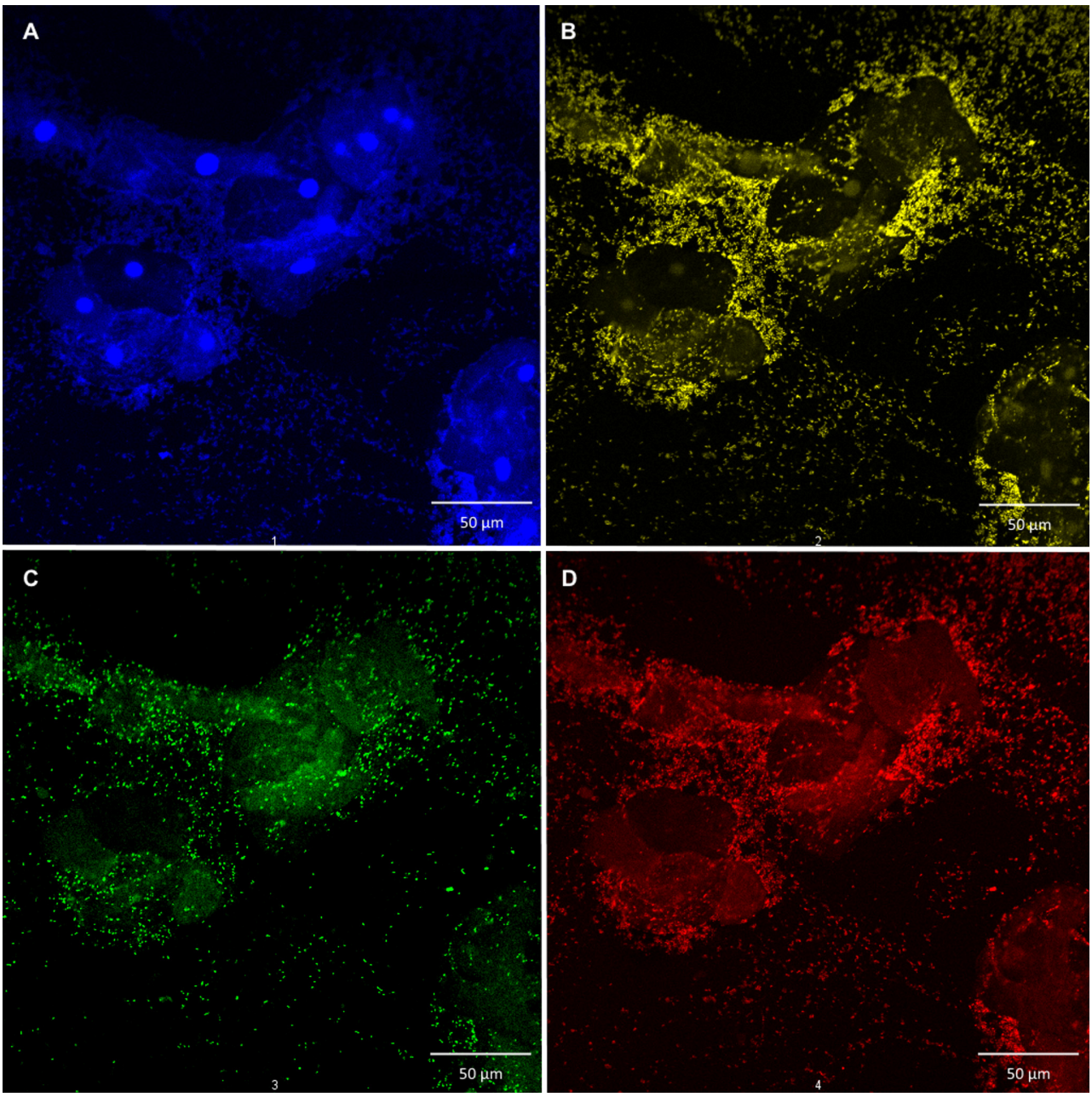

Fig 5. Polymicrobial biofilm of *A. vaginae* and *G. vaginalis* in different panes. Confocal laser scanning image with 400 x magnification of polymicrobial biofilm in different panes, A: vaginal epithelial cells DAPI in blue, B: all bacteria, BacUni-1 PNA-probe with Alexa Fluor 555 in yellow, C: *A. vaginae* specific PNA-probe AtoITM1 with Alexa Fluor 488 in green, D: *G. vaginalis* specific PNA-probe Gard162 with Alexa Fluor 647 in red (superimposed image can be seen in Fig 3A).

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ionic-strength conditions compared to DNA probes [40]. Low ionic-strength conditions prevent the complementary genomic sequences from reannealing when performing the FISH procedures; they facilitate denaturation of RNA secondary structures and favors hybridization of the PNA probes with nucleic acids. In combination with the superior penetration of PNA probes through the cell wall and hydrophobic bilayer of the target organism [41], PNA FISH is

Table 3. Performance of *A. vaginae* (AtoITM1) and *G. vaginalis* (Gard162) PNA probes, compared to qPCR results, for 119 vaginal slides.

FISH	qPCR				
	***A. vaginae* positive**	***A. vaginae* negative**	***G. vaginalis* positive**	***G. vaginalis* negative**	**Total**
AtoITM1 positive	48 (66.7%)	5 (10.6%)			53
AtoITM1 negative	24 (33.3%)	42 (89.4%)			66
Gard162 positive			82 (86.3%)	6 (25%)	88
Gard162 negative			13 (13.7%)	18 (75%)	31
Total	72	47	95	24	

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a fast, simple and robust assay. We compared DNA and PNA probes (data not reported) and can confirm that PNA FISH is faster and more robust than DNA FISH. In the current study, PNA FISH proved to be highly efficient for the identification and visualization of the spatial arrangement of *A. vaginae* and *G. vaginalis* in the BV-associated biofilms. Moreover, PNA FISH showed excellent inter-assay repeatability for all three probes used.

PNA FISH probe performance on bacterial strains

For the design of the PNA probes, we opted for Alexa fluorochromes, which have similar spectral properties as other fluorochromes, such as cyanine dyes, but are brighter and more resistant to photo bleaching [42].

PNA probes that specifically target *A. vaginae* have not been described before. The only probe thus far reported was a DNA probe (Ato291) [27]. The probe was designed to detect bacteria belonging to the *Atopobium* cluster in fecal samples. However, *in silico* evaluation of the specificity of the Ato291 probe showed cross-hybridization with other bacteria belonging to the Coriobacteriaceae, a family of Actinobacteria, to which *A. vaginae* belongs. This is not surprising, since the probe was originally designed on the basis of sequences of Coriobacteriaceae strains isolated from feces and clinical material. The probe has been used for the detection of *A. vaginae* in vaginal samples by Swidsinski et al. [9], but their findings have not yet been confirmed by other groups. In our experiments, using a PNA equivalent of the Ato291 probe, we showed a low specificity of the Ato291 probe on vaginal clinical isolates. The Ato291 probe

Table 4. Presence of *A. vaginae* and *G. vaginalis*, as assessed by FISH, in relation to *A. vaginae* and *G. vaginalis* loads as determined by qPCR for 119 vaginal samples.

Detected by PNA FISH	Total	***G. vaginalis* count 0** N (%)	***G. vaginalis* count <10^6 geq/ml** N (%)	***G. vaginalis* count >10^6 geq/ml** N (%)	geq/ml	***A. vaginae* count 0** N (%)	***A. vaginae* count <10^6 geq/ml** N (%)	***A. vaginae* count >10^6 geq/ml** N (%)	***A. vaginae* mean log** geq/ml
A. vaginae									
Absent	66	20 (30.3%)	13 (19.7%)	33 (50.0%)	4.87	42 (63.64%)	7 (10.61%)	17 (25.76%)	2.55
Dispersed only	27	3 (11.1%)	0 (0.0%)	24 (88.9%)	6.82	3 (11.11%)	4 (14.81%)	20 (74.07%)	6.21
Biofilm[1]	26	1 (3.9%)	1 (11.8%)	24 (92.3%)	7.50	2 (7.69%)	0 (0%)	24 (92.31%)	7.66
G. vaginalis									
Absent	30	17 (56.7%)	6 (20.0%)	7 (23.3%)	2.57	24 (80.00%)	3 (10.00%)	3 (10.10%)	1.05
Dispersed only	31	2 (6.5%)	6 (19.4%)	23 (74.2%)	6.68	13 (41.94%)	5 (16.13%)	13 (41.94%)	3.97
Biofilm	58	5 (8.6%)	2 (3.5%)	51 (87.9%)	7.18	10 (17.24%)	3 (5.17%)	45 (77.59%)	6.55

[1]*A. vaginae* biofilm = polymicrobial biofilm consisting of *A. vaginae* and *G. vaginalis*; No slides had *A. vaginae* biofilm only.

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cross-hybridized with three out of five *G. vaginalis* strains and all five *Lactobacillus* species. Therefore, we designed two new probes for *A. vaginae* targeting the 16S rRNA-gene, based on published PCR primers [28,29] and we adjusted the sequences to fit the requirements for PNA probes. One of the new probes, AtoITM1, did not cross-react to any of the tested species and was further used for detection of *A. vaginae* in vaginal slides.

Gard162 is the first PNA FISH probe designed specifically for *G. vaginalis* and has extensively been tested by Machado et al. on a variety of cultured bacterial strains and clinical samples [30,31]. Using this probe, we obtained clear hybridization for all *G. vaginalis* isolates tested and observed no cross-reaction with strains of the other species tested, confirming the findings of Machado et al. [30].

PNA FISH probe performance on clinical samples

Vaginal slides proved to be a valid sample type for imaging of the composition of the vaginal microbiome, if processed directly after sampling, as shown by Peltroche-Llacsahuanga et al. [43]. Collection of a vaginal swab is an easy and cheap sampling method, with a minimal burden on the study participant or patient. After heat fixation, we recorded that the slides can be stored at room temperature for up to at least six months and can be easily transported. A high quality vaginal sample can be obtained by thinly rolling the swab onto the slide. A thick vaginal 'smear' on the contrary where the material is smeared onto the slide is not useful for FISH and microscopic visualization.

The probes were also applied to 119 vaginal slides from women for whom the bacterial loads of *G. vaginalis* and *A. vaginae* had been quantified by qPCR. qPCR was used in this study as the reference method to evaluate the performance of the FISH probes, although comparison of these methods is subject to some limitations. qPCR is highly sensitive and was performed on a homogenized DNA extract representing half of the full sample. FISH however was carried out on 0.5 mm^2 of a vaginal slide, which could be heterogeneous. This is inevitably an underrepresentation of the vaginal sample. Both techniques were also performed using two different vaginal swabs, but the first collected specimen was used to prepare the slide as per study protocol.

After hybridization for 60 minutes and washing for 15 minutes, both at 60°C, the AtoITM1 probe gave only five false positive results, compared to qPCR results, resulting in a specificity of 90% for this set of samples However, 24 samples that were positive according to the qPCR were not detected by FISH, which gives a sensitivity of 67%. These results are comparable to the sensitivity observed for vaginal samples using FISH for detection of Group B *Streptococcus* [43]. The authors of this study obtained a higher sensitivity after extraction of the swabs by centrifugation of the swab head in a NaCl solution [43], but this method would probably disrupt the epithelial biofilm and thus prevent us from investigating the organization of the bacterial biofilm.

The relatively low sensitivity of the *A. vaginae* FISH assay cannot be explained by the bacterial load as measured by qPCR; the mean log of the true positive and false negative samples was not significantly different (log 7.73 and 7.19 geq/ml respectively). One possible explanation could be the typical structure of a biofilm, whereby an oxygen gradient exists from the top to the center of the biofilm [44]. Anaerobic bacteria such as *A. vaginae* are possibly found more embedded in the biofilm, to take advantage of the anaerobicity. It could be that the PNA probes are not able to fully penetrate into the inner parts of the biofilm; or that if the PNA probes do penetrate, the fluorescence could be masked and not detected due to low resolution of the equipment.

For *G. vaginalis*, 6 false positive results and 13 false negative results were found compared to 95 positive and 24 negative samples according to qPCR. This implies a sensitivity of 86% and specificity 75% for the detection of *G. vaginalis* by the Gard162 probe using our FISH protocol

for this set of samples. This is lower than reported by Machado et al. [30], who, using the same probe, showed a full agreement between qPCR and FISH results for 13 vaginal samples. We were not able to elucidate why these discordant results were obtained.

Bacterial loads and the presence of a biofilm

Our study shows that higher bacterial loads of *G. vaginalis* and *A. vaginae*, as detected by qPCR, are associated with a higher probability of presence of a bacterial biofilm. Both bacterial species are important constituents of the vaginal epithelial biofilm [9,45]. No samples contained *A. vaginae* in the absence of *G. vaginalis*, but almost half of the *G. vaginalis*-positive samples did not contain *A. vaginae* according to FISH results. Both bacteria were seen in a dispersed and an adherent state, but *A. vaginae* was always accompanied by *G. vaginalis*. The mere presence of *A. vaginae* did not simply predispose to a polymicrobial biofilm, but when *A. vaginae* was part of the biofilm, compared to a biofilm of only *G. vaginalis*, both bacterial species were present in higher concentrations.

We hypothesize that *G. vaginalis* is one of the main initiators of a vaginal biofilm, when it is present in high amounts. This vaginal biofilm creates a favorable environment for anaerobic bacteria, such as *A. vaginae*. One reason for the appearance of *A. vaginae* may be the presence of an oxygen gradient within the biofilm. By embedding itself within the biofilm, *A. vaginae* can take advantage of the anaerobicity, proliferates and exists in a mutualistic relationship with *G. vaginalis*.

Conclusion

Our study confirms that PNA FISH is a valuable tool for detecting and visualizing biofilm-associated organisms in vaginal slides. This study describes the design and evaluation of a new PNA probe, AtoITM1, which can be included in multiplex FISH in BV biofilm research. Using the new probe, we have demonstrated the presence of a polymicrobial biofilm, with *A. vaginae* taking part in a *G. vaginalis* dominated biofilm.

Supporting Information

S1 Protocol. Final Approved Protocol for "The Ring Plus project: Safety and acceptability of vaginal rings that protect women from unintented pregenancy" version 2.0, 16 April 2013.
(PDF)

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Author Contributions

Conceived and designed the experiments: LH VJ TC. Performed the experiments: LH ND LM VM. Analyzed the data: VJ LH. Contributed reagents/materials/analysis tools: MV TC. Wrote the paper: LH VJ ND LM VM MV TC.

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ORIGINAL ARTICLE

A fruitful alliance: the synergy between *Atopobium vaginae* and *Gardnerella vaginalis* in bacterial vaginosis-associated biofilm

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ABSTRACT

Objectives Bacterial vaginosis (BV) is characterised by a change in the microbial composition of the vagina. The BV-associated organisms outnumber the health-associated *Lactobacillus* species and form a polymicrobial biofilm on the vaginal epithelium, possibly explaining the difficulties with antibiotic treatment. A better understanding of vaginal biofilm with emphasis on *Atopobium vaginae* and *Gardnerella vaginalis* may contribute to a better diagnosis and treatment of BV.

Methods To this purpose, we evaluated the association between the presence of both bacteria by fluorescence in situ hybridisation (FISH) and BV by Nugent scoring in 463 vaginal slides of 120 participants participating in a clinical trial in Rwanda.

Results A bacterial biofilm was detected in half of the samples using a universal bacterial probe. The biofilm contained *A. vaginae* in 54.1% and *G. vaginalis* in 82.0% of the samples. *A. vaginae* was accompanied by *G. vaginalis* in 99.5% of samples. The odds of having a Nugent score above 4 were increased for samples with dispersed *G. vaginalis* and/or *A. vaginae* present (OR 4.5; CI 2 to 10.3). The probability of having a high Nugent score was even higher when a combination of adherent *G. vaginalis* and dispersed *A. vaginae* was visualised (OR 75.6; CI 13.3 to 429.5) and highest when both bacteria were part of the biofilm (OR 119; CI 39.9 to 360.8).

Conclusions Our study, although not comprehensive at studying the polymicrobial biofilm in BV, provided a strong indication towards the importance of *A. vaginae* and the symbiosis of *A. vaginae* and *G. vaginalis* in this biofilm.

Trial registration number NCT01796613.

INTRODUCTION

Bacterial vaginosis (BV) is the most prevalent vaginal disorder in women of reproductive age. It increases the risk of acquisition and transmission of sexually transmitted infections, including HIV, and is associated with preterm birth in pregnant women.[1–3] The condition is characterised by a change in the microbial composition of the vagina: the *Lactobacillus* spp., associated with a healthy vaginal microbiome, are outnumbered by micro-aerophilic and anaerobic organisms, including *Gardnerella vaginalis*.[3–7] The mere presence of *G. vaginalis*, however, is not sufficient for the diagnosis of BV using traditional diagnostic algorithms (see below) because many women without BV also have *G. vaginalis* in their vaginal microbiome.[4] BV is, however, associated with high counts of *G. vaginalis* using molecular methods and/or the presence of a *G. vaginalis*-containing polymicrobial biofilm.[4–10] Due to its strong adherence to vaginal epithelial cells and biofilm-forming capacities, it has been suggested that *G. vaginalis* initiates the colonisation of the vaginal epithelium and serves as a scaffolding to which other species subsequently can attach.[10–12]

One of the species that might attach to the biofilm initiated by *G. vaginalis* could be *Atopobium vaginae*.[13–14] Several molecular studies have indicated a probable role for *A. vaginae* in BV,[14–16] and it has also been suggested that *A. vaginae* plays a major part in the establishment of a biofilm, together with *G. vaginalis*.[9 10] Considering it has been found in 80–90% of cases of relapse[17] and some strains have been shown in vitro to be metronidazole resistant,[18] it could be of importance in the recurrence of BV after standard treatment with metronidazole.

The current gold standard in BV research is the microscopic evaluation and scoring of vaginal slides according to Nugent.[19] The diagnosis of BV is based on the absence of lactobacilli and the presence of small Gram-negative to Gram-variable rods (*G. vaginalis* and *Bacteroides* spp. morphotypes) and curved Gram-negative rods (*Mobiluncus* spp. morphotypes). In fact, bacterial biofilm can also be seen with this method in the form of clue cells, which are vaginal epithelial cells covered by layers of adherent Gram-negative and/or Gram-variable cells, that is, biofilms.[20] Using Gram staining, it is impossible to distinguish between the different bacterial species in the biofilm. By labelling the cells with a fluorescent probe, using fluorescence in situ hybridisation (FISH), the structure and composition of the biofilm can be studied in more detail.

To study the potential role of *A. vaginae* and the synergy between *A. vaginae* and *G. vaginalis* in the biofilm, we used our newly developed peptide nucleic acid (PNA) *A. vaginae* probe[11] together with an existing probe for *G. vaginalis*[21] and a universal bacterial probe[22] to investigate the composition of vaginal biofilm and its importance in BV.

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MATERIALS AND METHODS

Clinical samples

Vaginal sample collection and preparation

Vaginal samples were collected from 120 women participating in a clinical trial at Rinda Ubuzima in Kigali, Rwanda, studying the safety and acceptability of a contraceptive vaginal ring (Nuvaring), including the effect of the vaginal ring on the vaginal microbiome (the Ring Plus study—Clinicaltrials.gov NCT01796613).[23] Participants were between 18 and 35 years old and provided written informed consent for participation in the study. Depending on the group (continuous or intermittent ring use) to which the participant was randomised, a total of four or five samples from the same participant were taken over a period of four menstrual cycles. A total of 463 samples were analysed after Gram stain and after FISH using light microscopy and confocal laser scanning microscopy (CLSM), respectively.

Vaginal sampling was carried out by the study physician during a speculum examination in the Rinda Ubuzima research clinic. A cotton swab was brushed against the lateral walls of the vagina and was transported in its container to the Rinda Ubuzima laboratory within 20 min. Upon arrival in the laboratory, the swab was used to prepare a vaginal slide on a regular glass slide for Gram stain and a second vaginal slide on a Superfrost Plus slide (Menzel-Gläser, Braunschweig, Germany). All slides were air-dried, heat-fixed by passing through a flame twice and then stored in their appropriate boxes until Gram staining and/or shipment for FISH. The first slide was Gram stained and examined on-site in the Rinda Ubuzima laboratory in Kigali. The Superfrost Plus slides were stored and shipped at room temperature to the ITM where they were fixed for a minimum of 12 h in Carnoy solution (6:3:1, ethanol:chloroform:glacial acetic acid).[11]

Microbiological analysis of the vaginal samples

Peptide nucleic acid fluorescence in situ hybridisation

PNA FISH was performed as described earlier[11] using species-specific probes for *A. vaginae* (AtoITM1) and *G. vaginalis* (Gard162) and the broad-range BacUni-1 probe. The hybridised samples were stored in the dark at room temperature for a maximum of 1 week before microscopic observation, using CLSM (LSM700, Zeiss, Oberkochen, Germany). Detection and identification of individual bacteria were done at 400× magnification (objective: Plan-Apochromat 40x/1.3 Oil Ph3 M27). Separate scattered bacterial cells were defined as dispersed bacteria. Aggregates of bacterial cells, sticking to the vaginal epithelial cells, were defined as adherent bacteria forming a biofilm. The species-specific signal was considered positive only if it had a positive counterpart in the 4',6-diamidino-2-phenylindole (DAPI) stain and if it displayed a positive signal simultaneously with the universal probe. Semi-quantification was done for the dispersed and adherent bacteria in three categories (absent, present in low amount, present in high amount), but for the analysis only two categories (absent or present) have been used.

Nugent score

The status of the vaginal microbiome was assessed at the Rinda Ubuzima laboratory by Nugent scoring of a Gram stained vaginal slide.[19] A score of 0–3 was considered normal vaginal microbiome; a score of 4–6 intermediate microbiome and a score of 7–10 BV.

Statistical analysis

The clinical study sample size calculation was based on the primary objective to assess the pre–post changes in the vaginal microbiome and required 60 women in each group to require 95% power to detect clinically important changes in bacterial counts.[23] Data analysis was done using STATA10 (StataCorp LP, Texas, USA). While the samples were collected longitudinally, they were analysed cross-sectionally, with each sample as the unit of analysis. To study the association between the presence and absence of dispersed and/or adherent *A. vaginae* and adherent *G. vaginalis* in relation to BV status, we categorised the samples into five categories (table 1) based on combinations of the presence of both bacteria in dispersed and/or adherent form as visualised by FISH. To increase the statistical power, we made the vaginal microbiome status binary: Nugent score 0–3 (reference group) versus Nugent score 4–10 (table 2). A mixed-effects logistic regression model was fitted with BV as the binary outcome (ie, Nugent 0–3 vs Nugent 4–10) and biofilm characteristics as the main dependent variable. The model was adjusted for woman, randomisation group and study visit, because multiple samples per woman at multiple study visits were included in the analysis. ORs are reported with 95% CI and the p values are from χ^2 tests (table 2).

RESULTS

Characterisation of vaginal samples

In total, 463 of 527 samples from 120 women were available for FISH analysis, excluding 13 missing samples and 51 samples not readable due to the absence of epithelial cells on the slides. In all 463 samples, a positive signal was detected for the universal BacUni-1 probe. In 230 samples (49.7%), only dispersed bacteria were present, while the other 233 slides (50.3%) contained adherent bacteria as well (table 1). *A. vaginae* and *G. vaginalis* were part of this biofilm in 126 (54.1%) and 191 (82.0%) samples, respectively. Next, we visualised *A. vaginae* with FISH

Table 1 *Gardnerella vaginalis, Atopobium vaginae* and *G. vaginalis* with *A. vaginae* combinations for samples analysed with fluorescence in situ hybridisation (FISH) by absent, dispersed only and adherent ±dispersed category and stratified by Nugent scoring

	Total	Nugent 0–3	Nugent 4–6	Nugent 7–10
	N	N (%)	N (%)	N (%)
FISH all bacteria				
Absent	0	0 (0.0)	0 (0.0)	0 (0.0)
Dispersed only	230	197 (76.0)	19 (39.6)	14 (9.0)
Adherent ± dispersed	233	62 (24.0)	29 (60.4)	142 (91.0)
FISH *A. vaginae* (Av)				
Absent	268	201 (77.6)	24 (50.0)	43 (27.6)
Dispersed only	69	41 (15.8)	10 (20.8)	18 (11.5)
Adherent ± dispersed	126	17 (6.6)	14 (29.2)	95 (60.9)
FISH *G. vaginalis* (Gv)				
Absent	172	155 (59.8)	8 (16.7)	9 (5.8)
Dispersed only	100	71 (27.4)	15 (31.2)	14 (9.0)
Adherent ± dispersed	191	33 (12.8)	25 (52.1)	133 (85.2)
FISH Av and Gv combined				
Gv and Av absent	170	153 (59.1)	8 (16.7)	9 (5.7)
Gv or Av dispersed only	101	72 (27.8)	15 (31.2)	14 (9.0)
Gv adherent ± Gv dispersed and Av absent	51	14 (5.4)	8 (16.7)	29 (18.6)
Gv adherent ± Gv dispersed and Av dispersed	15	3 (1.1)	3 (6.2)	9 (5.8)
Gv and Av adherent ± Gv and Av dispersed	126	17 (6.6)	14 (29.2)	95 (60.9)

Table 2 Association between the bacterial presence of *Atopobium vaginae* and *Gardnerella vaginalis* by fluorescence in situ hybridisation (FISH) and the vaginal microbiome defined by Nugent scoring

G. vaginalis and *A. vaginae* combination	absent	*G. vaginalis* (Gv) or *A. vaginae* (Av) dispersed only	Gv adherent ± Gv dispersed and Av absent	Gv adherent ± Gv dispersed and Av dispersed only	Gv and Av adherent ± dispersed Gv and Av
Total=463	170	101	51	15	126
Nugent 0–3	153 (90)	72 (71.3)	14 (27.5)	3 (20)	17 (13.5)
Nugent 4–10	17 (10)	29 (28.7)	37 (72.5)	12 (80)	109 (86.5)
OR (CI)*	Reference	4.5 (2 to 10.3)	49.2 (15.9 to 151.8)	75.6 (13.3 to 429.5)	119 (39.9 to 360.8)
p Value χ^2 test*		0.001	<0.001	<0.001	<0.001

*The mixed-effects logistic regression model was adjusted for woman, randomisation group and visit.

in 195 (42.1%) samples; in 69 samples (14.9% of the total 463 samples) *A. vaginae* was present in a dispersed state, whereas in 126 samples (27.2%) the *A. vaginae* bacteria were seen adherent to epithelial cells (table 1). For 122 (97.0%) of the samples with adherent *A. vaginae*, concurrent dispersed *A. vaginae* bacteria were observed. *G. vaginalis* was detected by FISH in 291 (62.9%) samples; it was detected as dispersed-only *G. vaginalis* in 100 samples (21.6% of the total 463 samples) and for the remaining 191 samples (41.3%) *G. vaginalis* was adherent to the epithelial cells. Furthermore, when combining the results of both bacteria and considering only the 291 *G. vaginalis* FISH-positive samples, *A. vaginae* was absent in 98 of the slides (33.7%). On the contrary, only two (0.5%) of the 195 samples showing *A. vaginae* (dispersed and/or adherent) with FISH were negative for *G. vaginalis*; this included one sample with adherent *A. vaginae*.

One-third of the vaginal samples (n=156; 33.7%) was classified as Nugent score 7–10, 10% as Nugent score 4–6 (n=48; 10.4%) and the remaining 259 samples (55.9%) as Nugent 0–3. The majority of the samples without *A. vaginae* (n=201; 75.0%) and without *G. vaginalis* (n=155; 90.1%) were categorised as Nugent 0–3, thus indicating a healthy microbiome. A BV microbiome, defined by a Nugent 7–10 category, was present in 75.4% of samples with adherent *A. vaginae* (n=95) and in 69.6% of the slides with adherent *G. vaginalis* (n=133). In case of absent *G. vaginalis* and *A. vaginae* by FISH (n=170, 36.7%), a healthy microbiome (Nugent 0–3) was observed for 90.0% of the 170 samples (n=153). Furthermore, when considering *G. vaginalis* and *A. vaginae* adherent samples only (n=126), 75.4% of the samples were categorised as BV (Nugent 7–10) (FISH experiments in figure 1; table 1).

The presence of *A. vaginae*, *G. vaginalis* and combinations of both bacteria in dispersed and adherent forms in relation to BV status

The group of FISH samples without *A. vaginae* and *G. vaginalis* was used as the reference group (table 2). Compared with this reference group, the odds of having a Nugent score of 4–10 were increased when one or both bacteria were present in the dispersed state without adhering to the vaginal epithelium (OR 4.5 (CI 2 to 10.3)); it was increased further when *G. vaginalis* was part of an adherent biofilm on the epithelium (OR 49.2 (CI 15.9 to 151.8)) and even more when dispersed *A. vaginae* accompanied this *G. vaginalis* biofilm (OR 75.6 (CI 13.3 to 429.5)); ultimately the OR was highest when *A. vaginae* was part of the *G. vaginalis* biofilm as well (OR 119 (CI 39.9 to 360.8)).

DISCUSSION

We set out to study the potential role of *A. vaginae* in BV and the synergy between *A. vaginae* and *G. vaginalis* in the BV-associated biofilm.

Our study confirms that both *A. vaginae* and *G. vaginalis* are important constituents of the vaginal epithelial biofilm.[9 11] Adherent *A. vaginae* and *G. vaginalis* were visualised in, respectively, 54.1% and 82.0% of samples with bacterial biofilm (detected using the universal BacUni-1 probe), suggesting an important role for both bacteria in this polymicrobial biofilm. Using FISH, we only found two samples containing *A. vaginae* (dispersed in both, adherent in one) in the absence of *G. vaginalis*, while more than one-third of the *G. vaginalis*-positive samples was negative for *A. vaginae*. This is in accordance with prior reports on the association of *A. vaginae* with *G. vaginalis*.[9 11 15 16 24] We showed that the presence of both bacteria in the samples, regardless of their existence in a biofilm, was associated with an elevated or high Nugent score indicative for vaginal dysbiosis and BV. The highest probability of having a Nugent score higher than 3 was seen when both *A. vaginae* and *G. vaginalis* were part of a biofilm attaching to the vaginal epithelial cells.

The association of *G. vaginalis* with BV was originally described in 1954 by Gardner and Dukes.[25] The involvement of *A. vaginae* in BV, however, has only been established 10 years ago.[13–15] Swidsinski *et al*[9] found vaginal biopsies with vaginal biofilm to be positive for *G. vaginalis* and *A. vaginae* when using fluorescent probes, although in our hands this *A. vaginae* probe cross-reacted with other vaginal species as well.[11]

The presence of *A. vaginae* in the BV-associated biofilm could have a major impact on treatment. Susceptibility to metronidazole, the standard treatment for BV, varied significantly across various *A. vaginae* strains in vitro.[18] In vivo data are scarce, but Bradshaw *et al*[17] found that rates of recurrence of BV were higher when *A. vaginae* was present in the vaginal microbiome in addition to *G. vaginalis*. In another study with topical metronidazole gel by Ferris *et al*,[13] it was shown that a high concentration of *A. vaginae* before treatment was associated with complete or partial failure of treatment for BV. In the above studies, no distinction was made between dispersed and biofilm-associated bacteria. Nevertheless, as bacteria in a biofilm are less sensitive to antibiotic treatment[26] and considering the evidence from our study that the formation of a bacterial biofilm is more likely to occur when *A. vaginae* is present in the vaginal microbiome, future design of studies may want to take this distinction into account when treating BV.

Our study has shed new light on the significance of *A. vaginae* and the synergy between *A. vaginae* and *G. vaginalis* in vaginal dysbiosis, using highly specific PNA probes for both bacteria. However, a limitation was that we used multiple samples from the 120 women of the Ring Plus study. Ideally, we should repeat the study in a larger group of women. Furthermore, although we assessed the association between bacterial biofilm and vaginal dysbiosis, more research is needed to

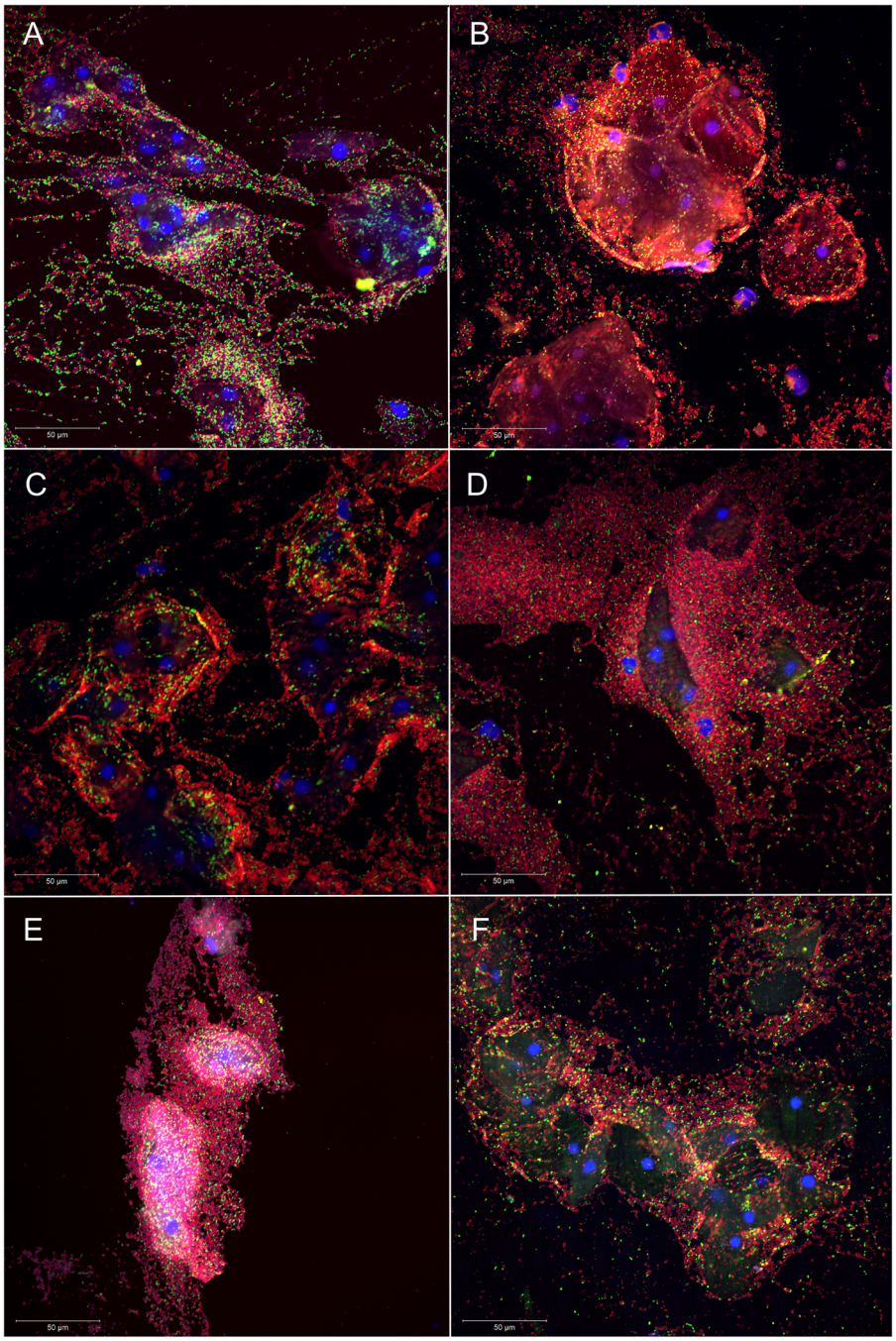

Figure 1 Superimposed confocal laser scanning images with 400× magnification of *Atopobium vaginae* +*Gardnerella vaginalis* biofilm in six vaginal samples (A–F): vaginal epithelial cells DAPI in blue, *A. vaginae*-specific peptide nucleic acid (PNA)-probe AtoITM1 with Alexa Fluor 488 in green and *G. vaginalis*-specific PNA-probe Gard162 with Alexa Fluor 647 in red. For clarity, we omitted the BacUni-1 plane, such that the bacteria that did not hybridise with Gard162 and AtoITM1 are visible in DAPI blue only.

unravel the exact mechanisms of biofilm formation in BV, including the role and the importance of both bacteria studied, to finally define improved regimens for treatment of BV. Moreover, since BV is a polymicrobial condition, new research should study the involvement of other bacteria related to BV.

In conclusion, the presented study uncovered a key piece of the BV puzzle confirming first, the importance of *A. vaginae* in BV-associated biofilm and second, showing the joint presence of *A. vaginae* and *G. vaginalis* in a biofilm. Future studies covering a wide array of BV-associated bacteria may help to further delineate biofilm mechanisms in BV.

Key messages

- This study shows that *Atopobium vaginae* is an important constituent of the vaginal biofilm, and is of relevance in the context of bacterial vaginosis (BV).
- We show that *A. vaginae* is almost always accompanied by *Gardnerella vaginalis* in BV, but that *G. vaginalis* can be found without *A. vaginae* in the vaginal microbiome.
- By tackling constituents of the biofilm, the above knowledge can contribute to a more effective and goal-oriented treatment and improve women's reproductive health.

Handling editor Jackie A Cassell

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Contributors All authors were involved in the main study that generated the data. For the present study, LH wrote the first draft of the manuscript. VJ, TC, MV and JvdW revised and edited the text. IDB, TC, LH, VJ and JvdW created the experimental design. SA, LM, VM and LH performed the testing and VJ and LH performed the data analysis. All authors revised and approved the present version of the manuscript.

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Patient consent Obtained.

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A fruitful alliance: the synergy between *Atopobium vaginae* and *Gardnerella vaginalis* in bacterial vaginosis-associated biofilm

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